EDITOR'S VIEWPOINT

Kydd (p. 171) used strain gauges and the Wheatstone bridge to measure forces exerted by the tongue on a lower denture in one individual. The paper is designed primarily to report a technic for measuring tongue force and should offer a useful tool to measure forces on different types of dentures and under different circumstances. For example, one may learn whether tongue training might reduce the tendency to dislodge lower dentures.

Johnson (p. 175) reports on a study of blood loss in oral surgery. It is interesting that the quantity of blood lost in extensive oral surgical procedures may exceed that lost in many other operations usually considered as "major."

Finn (p. 185) found enamel from exfoliated deciduous teeth of children born in a city with artificially fluoridated water to be less soluble when powdered and agitated in acid solution than enamel from similar teeth in the same city before fluoridation and teeth from a city with relatively fluoride-free water. He suggests that his data show that fluorides from communal water supplies affect the surface layers of enamel, posteruptively, as they contact them whenever water is consumed. The fact that topical applications of 2 per cent fluoride solutions have any effect at all would support this hypothesis and the data from regions recently fluoridated and showing reduced caries rates in older children give further credence to it.

The possibility that an antienzymatic effect of fluoride might act, in part at least, as an inhibitor of dental caries has been projected on numerous occasions, but aside from showing antibacterial effects of fluorides in vitro at relatively high concentrations the antienzymatic mechanism has not been shown. Although Lilienthal and Martin (p. 189) found that artificially fluorosed powdered enamel inhibited acid production to a degree similar to CaF, they concluded that fluoride at the concentration expected at the tooth surface does not prevent acid formation. The search for the elusive mechanism of caries reduction by fluorides will continue.

Lilienthal (p. 197) was unable to demonstrate the inhibitory effect of low concentrations of fluorides on acid formation by oral bacteria. Fluorides inhibit acid production in pure cultures of lactobacillus and streptococci only after growth has ceased; during active growth 45 ppm of fluoride had no effect on acid production. The presence of calcium, orthophosphate, manganese, and magnesium results in different effects. While 32 ppm of fluoride had a slight inhibitory effect on acid production, addition of calcium completely reversed the inhibition while magnesium, manganese, and phosphate enhanced the inhibition. These effects are most important in considering the action of fluorides in saliva, or when added to dentifrices, mouthwashes, and other vehicles.

Peckham, Leopold, and Hess (p. 205) analyzed the fluoride in the organic and inorganic fractions of dentin and enamel of teeth from a high fluoride region (2.1 ppm). All, or most, of the fluorides appear to be associated with the inorganic portion.

Blackwell and Fosdick (p. 210) suggest that in addition to supplying the substrate to be reduced to acid, sucrose in the dentobacterial plaque may retard the rate of acid production. This hypothesis is based on their experiments showing that sucrose added to lactate buffer solutions decreases the solubility of powdered enamel in vitro. The authors recognize the need for further studies under conditions more analogous to those in the mouth. The possible roles of viscosity changes produced by the sucrose, coating effects, chelation and hydration effects are discussed.

Tullar (p. 220) used a simple in vitro technic to demonstrate the rapidity with which plaque or salivary sediment can convert sugars to acid demineralizing pHs (4.3

to 4.5) were produced in 15 to 210 seconds in plaque material and pH 4.75 was observed in salivary sediment in 8 to 190 seconds after sugar was added. Four individuals, one caries-free and one with practically no evidence of caries, of 37 tested did not produce acid in their salivary sediment. This demonstration of rapid acid production strengthens Stephan's observation of in vivo acid production in 3 to 30 minutes where salivary enzymes may be acting to reduce acid formation.

Mahler and Manly (p. 226) measured the pH drop from sugars in dentobacterial plaque material and in simulated plaques consisting of microorganisms in compact layers. In vitro, the synthetic plaques resulted in greater pH drops than the natural plaques, possibly due to lack of dilution with extraneous cells and other material in the instance of the synthetic plaques. Any of a number of the organisms of the plaque can produce significant pH drops. Because streptococci were consistently recovered from plaques and because they produced a terminal pH of 3.5, the authors suggest that they may be more important than lactobacilli. However, many factors not present in in vitro tests are operative in the mouth and no conclusions should be drawn until due consideration is given to the saliva, the cell enzymes, the nonplaque portions of the oral flora, and other "in vivo" conditions. This artificial plaque technic offers a screening device for study of the effect of various agents on the plaque without the diluting and detergent effect of saliva and free of its antibacterial activity.

Shockley, Randles, and Dodd (p. 233) tested the ability of lactobacilli, streptococci, and staphylococci to ferment sorbitol, glucose, and glycerol in vitro. Sorbitol was not fermented by most of the strains of organisms utilized, and even when fermentation occurred it was slow with little acid production. They conclude that sorbitol would not contribute to acid production significantly, and that there might be beneficial results of its use as a sweetening agent. If the intake of sweet-tasting substances cannot be controlled by educational methods, either an antiacid-producing agent might be added or nonacid-forming sweetening agents substituted. The use of sweetening agents other than glucose and sucrose has gained some favor in the interest of reduced caloric intake but their use to reduce caries might have been a more rational approach.

Turner and Anders (p. 241) tested the method used by Stack (*J. D. Res.* 33: 316, 1954) to identify tryptophane in saliva. Using this p-dimethylamino-benzaldehyde technic on known solutions of indole, indole 3-acetic acid, indole -3-propionic acid, tryptamine and trypophine, they found it inadequate for their differentiation in saliva. Determination of tryptophane and many other substances is difficult in saliva, leading to some neglect of their salivary concentrations, or to widely differing values reported by differing authors.

Rathje (p. 245) added pure hydroxyapatite to stimulated and unstimulated saliva of caries-resistant individuals. The resulting decrease in calcium and phosphate content of saliva suggests a possibility of remineralization of enamel by the saliva but, as the author points out, before such a phenomenon is accepted as factual, in vivo demonstration is necessary. Unfortunately, in the enthusiasm of a limited number of writers, they often overlook such warnings as that of Rathje and may use such in vitro evidences to support their unsupported hypotheses. Such aberrations are not limited to advertising writers but, in more subtle manner, may appear in reputable scientific journals. Editors and their consultants are not infallible in detecting these errors, for they are not familiar with the details of all of the literature nor do they wish to serve as censors.

A possible explanation of the enigma of reduced caries activity without comparable reduction in salivary lactobacillus counts when penicillin dentifrices are used is offered by Dreizen, Mosny, Paddor, and Spies (p. 249). They determined the minimal amount of penicillin needed to prevent growth of lactobacilli, in vitro, and then the amount needed to inhibit growth in the presence of yeasts. The results suggest that acid production by acidogens can be drastically reduced by penicillin in broth cultures containing yeast, although the acidogens continue to grow. Mechanisms of this character may explain the frequent observation that salivary

lactobacillus indices are not reliable indicators of caries' activity in mouths treated by some anticaries agents.

Battistone and Burnett report on the amino acid composition of human dentinal protein (p. 255) and on the amino acid composition of human enamel protein (p. 260). These 2 papers give the results of amino acid studies after teeth have been prepared by chelating agents. These agents allow analyses of enamel and dentin which has been decalcified at/or close to neutral pH. It appeared that such methods should have greater accuracy than older methods using relatively strong acids for decalcification. These analyses indicate that the organic portion of dentin is essentially collagenous and that enamel protein is not a typical keratin. This latter finding is contrary to the general concept of enamel organic matrix as a keratinous material.

Recognizing the need to maintain the amino acid composition intact during processing of teeth for analysis, Battistone and Burnett (p. 263) compared analyses after different methods of preparation. Storing, separating methods, and decalcification all effected the amino acids recovered from enamel and dentin.

Hess, Lee, and Peckham (p. 273) found 0.008 per cent of cholesterol in enamel, one-fourth the amount they had previously identified from dentin. The total lipids, other than cholesterol, was found to be 0.36 per cent in dentin and 0.10 per cent in enamel.

White and Hess (p. 276) were unable to demonstrate any soluble phosphatase in mature dentin although there appeared to be a small quantity of bound phosphatase, possibly with the collagen. Mature bones gave similar results. An activity of phosphatase-like nature could be obtained from dentin and bone after removal of the organic phase but the previously reported respiratory enzymes ("dehydrogenase" and "oxidase") could not be confirmed.

Shaw and Sweeney (p. 286) examined 1,401 albino rats and 1,314 cotton rats for caries distribution. No difference in caries susceptibility was observed between males and females in these species, unlike earlier findings in hamsters. As group bilateral similarity was observed for distribution of carious lesions but the bilateral similarity was much less pronounced in the caries-resistant animals. If this same relationship holds for humans, the technic of prophylactic restorations on one side when the first side is carious may have less validity in individuals who are caries resistant than in the susceptible.

Rosen, Ragheb, Hunt, and Hoppert (p. 291) isolated 100 strains of lactobacilli on tomato juice agar from rats of the Hunt-Hoppert colony and 100 were isolated on synthetic media. Most of those isolated on the tomato juice agar would not grow on the synthetic medium. The authors point out that different strains may be identified from the same source if different media are used. Unfortunately, efforts to differentiate lactobacilli from the resistant line from those isolated from the susceptible line was stopped when 15 strains from resistant animals on synthetic media and 22 on tomato juice were found "to be very similar to lactobacilli isolated from caries susceptible rats." It seems that a careful study of strains from these two groups should be undertaken before the search for differences is abandoned.

Schultz-Haudt and Scherp (p. 299) searched for chondrosulfatase production by microorganisms found in the gingival region. Since chondrosulfates form a part of the intercellular cementing substance of gingival tissue, it was believed that the demonstration of the enzyme which breaks it down might have some significance. Mixed cultures from the gingival sulcus produced chondrosulfatase, but pure cultures of the commonly recognized components of the gingival flora did not. A micro-aerophilic diphtheroid bacillus capable of chondrosulfatase production was isolated from the gingival flora. Certain symbiotic relationships were apparent. The possible role of this enzyme and hyaluronidase and beta-glucuronidase in breaking down the ground substance of gingiva is apparent. The authors point out that the presence of the organism in gingival crevices free of disease does not indicate that under favorable conditions the organism cannot produce significant quantities of enzyme to lead to gingival damage.

Zander (p. 308) presents a technic for experimental production of an "epithelial attachment-like" structure at the borders of an acetate strip implanted into the gingival

sulcus. Even the preliminary results suggest the need for careful re-evaluation of old and new concepts of epithelial attachment of the gingiva to the tooth.

Waerhaug (p. 313), who has renewed interest in the epithelial attachment and the enamel cuticle through investigations of attachment-like phenomena on nondental surfaces, studied the formation of "enamel cuticle" by an experimental approach. His investigations question the existence of primary and secondary cuticles as distinct entities and suggest that enamel cuticle may be formed by cells of the "enamel cuff," not solely by the ameloblasts. The studies are provocative and suggest an explanation for the enamel cuticle-like structure on cementum.

Waerhaug (p. 323), from studies over surfaces of teeth roughened by a diamond stone, concludes that roughness, per se, does not cause significant gingival inflammation but that rough surfaces afford places for bacteria to colonize in plaques. Since the bacteria and their toxins are irritants, the clinical effect is the same. Polishing of surfaces of restorations at or below the gingival margin is recommended.

Bernarde, Fabian, Rosen, Hoppert, and Hunt (p. 326) describe an apparatus to hold several rats while collecting saliva and the use of a sialogogue and an anesthetic to induce salivary flow and keep the animals controlled. While the method yields adequate saliva, the high mortality rate (10 per cent) suggests the need for continued search for a better-tolerated drug technic.

H. B. G. R.

QUANTITATIVE ANALYSIS OF FORCES OF THE TONGUE

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IN RECENT years there have been many reports[1, 2] submitted and devices constructed to measure the forces acting on the dental arch. Most of these were concerned with vertical forces, or occlusal biting forces.

The purpose of this experiment was to devise a technic to determine how much force the tongue could exert on a denture in an anterior direction in the area of the lower anterior teeth, and in a lateral direction on a lower denture in the second premolar–first molar area.

TESTING PRINCIPLE INVOLVED

The technic employed is a modification of the method used by Lissner and Gurdijian[3] and others,[4, 5] where a bonded SR-4 electric resistance strain gauge was used. The electric resistance strain gauge is based on the principle that if the cross section of a given wire is decreased, its ability to carry on an electric current is decreased, or the resistance of the wire is increased. The strain gauges used in this experiment were so placed that the muscular pressures exerted by the tongue would produce a deformation of the wire within the gauge, and therefore the total resistance of the gauge would be altered.

Utilized in this capacity, a change in the force exerted produces a deformation of the gauge wire which can be plotted as a linear, straight line. Such a plotting indicates that one derives an output of voltage from the proper external measuring circuit which is proportional to the dimensional change or force applied. It then is possible to find the constant that will be maintained for any other point in the range. Pressures outside the operating range will deviate from the linear constant and will be readily discernible.

For the external circuit the principle of a balanced, Wheatstone bridge was used with 3 known resistance values permitting the finding of the fourth. In this procedure the ratio of the arms are one to one, since the standard and unknown are both wire gauges within 0.2 ohm resistance of each other. An alternating current amplifier and a multichannel pen recorder were used to give dynamic as well as static recordings.

The test device was a lower full denture base that was occluded with an upper full denture base (Fig. 1). These bases were made of methyl methacrylate. The vertical dimension was obtained and maintained between the bases by 4 vertical rods imbedded in the lower denture base. These rods contacted the upper denture base at predetermined recessed areas.

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The recessed area in the upper denture base was determined by taking a centric relationship between the upper and lower jaws and transferring this relationship to an articulator. Then the upper and lower acrylic bite rims used in this experiment were mounted on the articulator in centric position. The holes for the reception of the rods were recessed in the upper denture, so that the denture would be retained in centric.

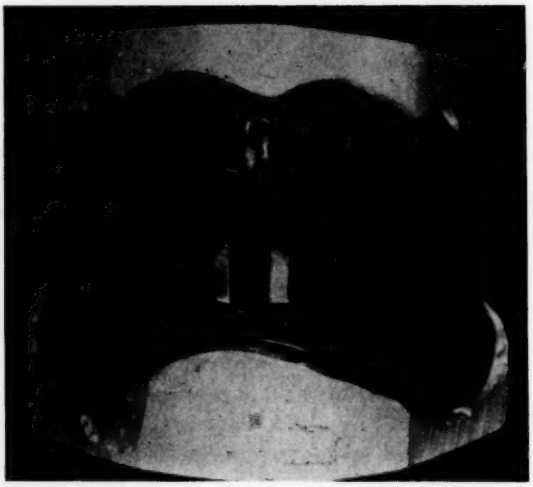

Fig. 1.—Testing device as it appears intraorally with strain gauge attached to milled labial block.

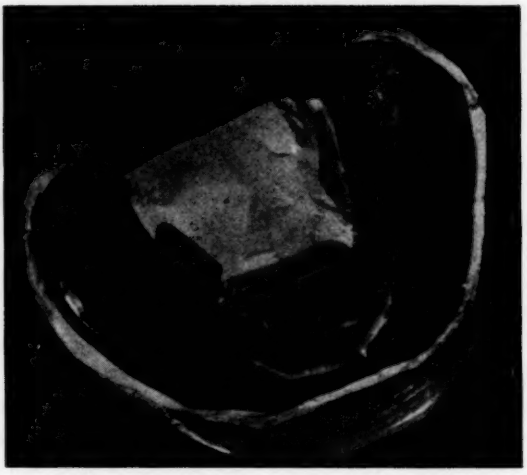

Fig. 2.—Position of rods, to maintain vertical dimension.

Between these rods were 3 carefully milled 15 mm. x 15 mm. x 2 mm. blocks of methyl methacrylate. The acrylic blocks were processed into the lower denture base but did not contact the upper base or the rods (Fig. 2).

These blocks were positioned to approximate the lingual surface of the lower incisor area and second premolar–first molar areas. To these blocks were attached electric resistance strain gauges. The gauges were attached to the labial and buccal aspect so that there would be no interference with the tongue. The acrylic blocks were recessed on the buccal to a depth of 0.5 mm. so that the gauges could be processed into the acrylic to assure a better bond between the gauges and blocks. Each of the blocks was separately calibrated outside of the mouth with known loads. The temperature was maintained at 98° F. during the calibration procedure to simulate the temperature that would be found in the test subject's mouth.

The material of the block and its thickness must be chosen for the range of loads it is expected to measure, for not only must the dimensional change be kept within certain limits dictated by the extensibility of the wire gauge, but the material used must "recover" or have a reversible action.

The blocks, therefore, were calibrated before and after any testing had been done to determine if the slow recovery period of methyl methacrylate had become apparent, as it usually does when a heavy stress is applied. Our tests showed no evidence of lack of recovery of the plastic, which would infer that the maximum load in relation to the size of the blocks was insufficient to cause permanent distortion of blocks.

TEST SUBJECT

The test subject was an edentulous 30-year-old man. The full upper and lower dentures he was using were removed and the testing device was placed in his mouth. The patient was asked to close his mouth for a few minutes so that the temperature of the testing device would be raised to approximately 98° F. Then the recorder was calibrated to a zero point when the tongue was at rest.

The patient was then asked to "bite down, and extend his tongue anteriorly with as much force as possible." After the force had been applied for 2 or 3 seconds the patient was told to relax his tongue. Then the patient was asked to extend his tongue in a lateral direction against the block in the left first molar–second premolar area. Again, after a few seconds' rest, the patient was asked to extend his tongue in the right lateral direction. After a few minutes' rest the cycle was resumed; tongue extended anteriorly, then left, then right. This regimen was continued until pressure had been applied to each of the 3 blocks 35 times. At this point the test was discontinued as fatigue was noticeable.

RESULTS

1. It was found that 5.2 pounds was the maximum that could be exerted anteriorly in the lower incisor area.

2. It was found that 2.6 pounds was the maximum that could be exerted laterally in the area of the right lower first molar–second premolar.

3. It was found that 2.3 pounds was the maximum that could be exerted laterally in the area of the lower left first molar–second premolar.

4. Since the findings are based on measurements from one individual the results are not considered necessarily valid for groups or for other individuals.

SUMMARY

1. A method of recording lateral and anterior forces exerted by the tongue is described.

2. The preliminary results obtained with this method on one individual showed 5.2 pounds maximum anterior thrust and 2.6 (right) and 2.3 (left) maximum lateral force.

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BLOOD LOSS IN ORAL SURGERY

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WITH STATISTICAL ANALYSIS BY RACHEL M. JENSS, D.Sc.*

THE problem of clinically evaluating reduced blood volume due to whole blood loss is one that has received little attention in oral surgical practice. So far as could be ascertained from rather exhaustive perusal of the literature, no scientific report has ever been published on this subject, although the literature is replete with articles on blood loss in all other branches of surgery. The exclusion of exodontia and oral surgery leads one to believe that the extent of hemorrhage associated with the extraction of teeth is generally accepted as insignificant.

The quantity of blood that escapes the vascular bed during a surgical procedure depends upon the size and the number of vessels surgically interrupted and the length of time bleeding or oozing is allowed to continue. The effect of hemorrhage on the host depends upon a number of factors, mainly upon the age and general health of the patient and the proportion of blood lost in relation to his normal blood volume.

In order to measure the extent of actual blood loss in oral surgical procedures, particularly exodontia with the aid of general anesthesia, this study was undertaken. Quantitative blood losses occurring in average dental patients during multiple or complete extractions and during alveolotomy were determined. For clinical evaluation the averages were compared with average blood losses in general surgical procedures. During the course of the investigation and subsequent analysis of the findings it became apparent that there may be another facet to this problem: blood loss tended to be materially affected by the surgical technic used.

CASE MATERIAL

Ninety-seven consecutive patients admitted to the Millard Fillmore Hospital by 8 oral surgeons for oral surgical treatment under general anesthesia between Feb. 12 and July 16, 1953, and an additional 82 patients admitted between Sept. 9, 1953, and April 1, 1954, were studied. Four were later excluded: 2 because of the presence of systemic disease and 2 because the anesthetic administered differed from the one used generally in the series. Exclusive of these cases, 175 patients are considered in the total hospital experience.

In the report[1] on the first series of patients, an additional 25 had been excluded from the statistical analysis: 12 whose blood pressures had not been recorded; 9 hypertensives; and 4 in whom it was believed the operative technic employed would require special grouping. However, upon completion of the second survey, that is, the latter 82 cases, and subsequent analysis of the entire series, no essential differences could be demonstrated, either as to rate or extent of hemorrhage, between normotensive and hypertensive patients. These patients

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are therefore included in the present study. Also, in the second survey the cases were compared as to the operative technic employed, but all are included in the total hospital experience.

Of the 175 patients 44.6 per cent were male and 55.4 per cent were female. Their ages ranged from 15 to 75 years, the average age for males being 40.8 years and for females 40.9 years. The indications for oral surgical intervention were variable, but periodontal disease was the most frequent. Pain was never the chief presenting complaint. The majority of these patients sought oral surgical treatment because their physician or dentist had requested such treatment for the eradication of potential foci of infection.

All patients were admitted to the hospital on the evening of the day preceding surgery. A complete history was obtained and preoperative urinalysis and complete blood count were made as a routine procedure of the hospital. A blood platelet count and bleeding, clotting, and prothrombin time tests were performed on a random sampling of patients in order to evaluate the clinical application of these tests in the present study. There were no polycythemic or anemic patients in the series. The hemoglobin content of the total group ranged from 12 to 16 Gm. per 100 c.c. of blood.

Preoperative blood volume determinations were also made on some patients. It would be ideal to have such data on all surgical patients but this is not always feasible, and normal values provide a practical guide, except in cases of extreme loss. A history of postextraction hemorrhage or bleeding tendency is perhaps the most significant finding to guide the oral surgeon in the proper management of the case. Such a history was elicited in 4 instances in this series.

METHOD

Since replacement by whole blood or other fluids should be instituted as soon as significant hemorrhage occurs or within as short a time thereafter as possible, it is of paramount importance for the surgeon to have a guide for estimating the volume or portion thereof to replace, yet the majority of methods for calculating blood loss are far too time-consuming for practical clinical application. The simplest solution to this problem is blood loss determinations made at the operating table or chair.

A gravimetric method was employed in this study. The procedure devised is fairly simple; it does, however, enlist the cooperation of the entire surgical team. An aspirator, a weighing scale recording in grams, a 500 ml. cylinder graduate, and from 200 to 300 ml. of sterile water are required in this method. The aspirator is used for suctioning blood as the hemorrhage occurs. The weighing scale is employed for the immediate recording of "used" sponge weights. The weight of dry, sterile sponges is remarkably constant, so that determining the quantity of blood contained in the used sponges is a simple mathematical calculation. The cylinder graduate measures a known volume of water for flushing the aspirator system to determine the amount of residual, coagulated blood contained in the suction tips and tubing. It also records the collected volume of blood within the aspirator bottle. This is then corrected by adding the lag volume of blood and subtracting the amount of water suctioned during the procedure.

The weight of the blood contained in the used sponges is considered equivalent to volume. Theoretically, the specific gravity of the patient's blood should be engaged in this conversion, but since, except for certain refined hematologic studies, the specific gravity of blood may be taken as unity, that figure was used. The total blood loss is the sum of the corrected quantity collected in the aspirator bottle and the converted blood weight from the used sponges.

This method does not take into account losses other than those of frank hemorrhage. Collection of blood on drapes, gowns, and gloves also was not considered. Bonica and Lyter,[2] in a survey of blood loss during general surgical operations, found that the amount of blood recovered from gloves, drapes, and gowns usually does not exceed 50 c.c.

The method as applied in the present study did, of necessity, include saliva as a part of the total measurable blood loss. Ideally, the salivary components should be subtracted, but a quantitative analysis of saliva present would not have been feasible because of the time

involved, the expense of the procedure, and the technical assistance required. However, in order to keep this factor to a minimum, each patient received premedication to reduce salivary secretions, and all throat packs were discarded, regardless of how much blood they contained. Also, hemorrhage into the tissues was not accounted for, and finally, the amount of hemorrhage occurring after the last suture was placed was not recorded. It is believed that these factors render insignificant the inclusion of resultant minimal saliva in the total calculated blood loss.

SURGICAL PROCEDURES

Alveolotomy was performed in 94.3 per cent of the 175 patients. The operative procedure included the removal of tori mandibularis and palatinus whenever necessary. If impacted teeth, regardless of their position or location, were removed during the course of a complete or multiple extraction, they were included under one of those two general headings.

The term "multiple extraction" refers to a subtotal odontectomy, whereas the term "complete extraction" refers to the removal of all remaining mandibular and maxillary teeth. A complete upper or lower extraction was included under the general heading of multiple extraction. No special categories were established to include gingivectomies, excision of hyperplastic growths, or biopsies, if these procedures were performed concomitantly with extraction. Removal of retained roots and foreign bodies was considered as extractions, and they were evaluated at the ratio of one to one.

Preclinical medication with morphine or pantopon and atropine or hyoscine, in individualized doses, was administered to all patients one hour preoperatively. Control of salivation in all but a few cases was excellent. In these few patients salivary activity was slight to moderate.

Pentothal sodium (2.5 per cent solution) supplemented with nitrous oxide–oxygen was the anesthetic universally employed. The quantity of Pentothal sodium used during the procedure was recorded in milligrams. Preoperatively and again postoperatively the blood pressure was ascertained. The time of starting and of completing the surgery was recorded. Finally, blood-loss studies were made at the termination of the surgery according to the gravimetric method described.

In 83 per cent of the patients nasopharyngeal tubes were inserted. The average dose of Pentothal sodium administered to these patients was 1,100 mg., based upon an average duration of surgery of 30 minutes. Endotracheal intubation was used in the remaining 17 per cent of the patients. The average requirement of Pentothal sodium in this group was 800 mg. Curare and/or succinylocholine was administered to all patients in this latter group.

In Fig. 1 the type of surgery performed is correlated with the number of patients in percentages. The surgery performed is based upon the number of extractions, and, as stated previously, includes under that general heading the removal of impactions, excisions of hyperplastic and hypertrophic growths, biopsy, and osseous corrections whenever performed concomitantly with extraction of teeth. The number of teeth removed is divided into groups of 8 in order to facilitate the analysis. The number of teeth extracted ranged from 1 to 30, and the average number of extractions per patient was 18.

The duration of the surgical procedures is shown in Fig. 2. The first column of the chart includes all of the procedures lasting less than 20 minutes; the second, third, and fourth columns represent 20-minute intervals; and column five includes all cases requiring more than 80 minutes for the procedure. The duration of surgery ranged from 7 to 90 minutes and averaged 30 minutes. Again the number of patients is shown as percentages of the total in each time group.

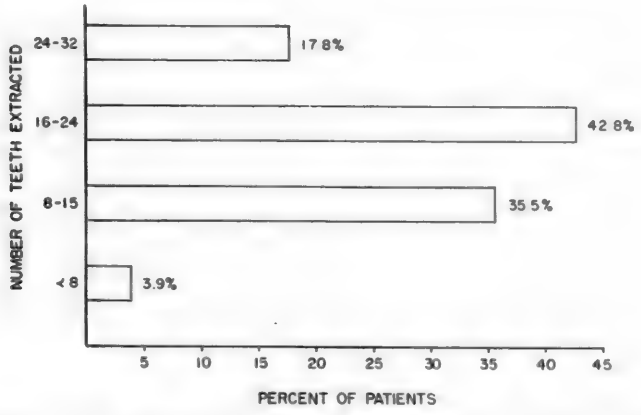

Fig. 1.—Type of oral surgery performed correlated with the number of patients (in percentages).

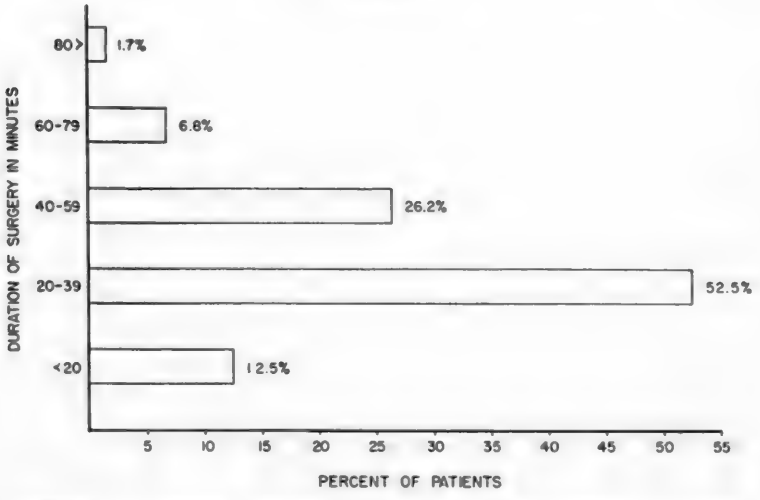

Fig. 2.—Duration of surgical procedures correlated with the number of patients (in percentages).

RESULTS

In Table I the relationship of blood loss to operating time and number of extractions in the total series of 175 cases is shown. Both the number and

per cent of patients are given in each blood-loss group. The surgery is divided into two categories: less than 16 teeth and 16 or more teeth extracted, with alveolotomy. The duration of surgery is also represented in two groups: less than 30 minutes and 30 or more minutes.

TABLE I
RELATIONSHIP OF BLOOD LOSS TO NUMBER OF EXTRACTIONS AND DURATION OF SURGERY
(TOTAL HOSPITAL EXPERIENCE)

BLOOD LOSS (ML.)	LESS THAN 16 TEETH EXTRACTED						16 OR MORE TEETH EXTRACTED					
	TOTAL CASES		DURATION OF SURGERY				TOTAL CASES		DURATION OF SURGERY			
			LESS THAN 30 MINUTES		30 MINUTES OR MORE				LESS THAN 30 MINUTES		30 MINUTES OR MORE	
	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
Under 100	19	25.6	17	36.9	2	7.2	5	4.9	2	9.4	3	3.7
100-199	29	39.2	21	45.6	8	28.5	23	22.8	12	57.3	11	13.7
200-299	9	12.2	3	6.6	6	21.5	28	27.8	5	23.8	23	28.8
300-399	15	20.3	5	10.9	10	35.7	20	19.8	2	9.5	18	22.5
400-499	2	2.7	—	—	2	7.1	9	8.9	—	—	9	11.3
500-599	—	—	—	—	—	—	7	6.9	—	—	7	8.7
600 and over*	—	—	—	—	—	—	9	8.9	—	—	9	11.3
Total	74	100.0	46	100.0	28	100.0	101	100.0	21	100.0	80	100.0

*Blood losses in this category were 620, 630, 677, 681, 720, 725, 822, 829, and 912 ml.

Blood loss in the total group ranged from 35 to 912 ml., the average loss being 223 ml. When the duration of the operation is compared with blood loss in the group with fewer than 16 extractions, 89 per cent operated upon in less than 30 minutes had a blood loss below 300 ml. If the duration of surgery surpassed 30 minutes, 57.2 per cent remained below the 300 ml. level, whereas 42.8 per cent sustained blood losses ranging from 300 to 500 ml.

In the group of patients who lost 16 or more teeth in the operative procedure, 44.5 per cent had blood losses above 300 ml. When the operating time remained below 30 minutes in this group, 90.5 per cent did not hemorrhage in excess of the 300 ml. level, but when the surgery exceeded 30 minutes in duration, 53.8 per cent of the patients sustained blood losses ranging from 300 to 912 ml. It was shown, both in the total hospital experience and when the data were analyzed according to groups of oral surgeons, that time is the most important consideration in the evaluation of blood loss. The more prolonged the operating time the greater the blood loss. In Fig. 3 blood loss is compared with per cent of the total patients.

No sex differences as regards blood loss could be demonstrated in this series, nor was there any correlation between age and blood loss.

No appreciable differences were found in either the rate or extent of hemorrhage between hypertensive and normotensive patients.

It was found that bleeding, clotting, and prothrombin times can be within normal limits in an individual who hemorrhages profusely during oral surgery. Conversely, positive results of these tests do not necessarily point to abnormal bleeding tendencies. Because false-positive results are frequent does not mean that the tests are without value, however, for even false-positive results will keep the surgeon alert to the possibility of severe hemorrhage. The most val-

uable guide in the prognosis of abnormal bleeding tendencies in the average dental patient was the history of previous episodes of excessive postextraction hemorrhage.

In general, if this study of 175 patients has any prognosticative value, it tends to show that when 16 or more teeth are extracted, with alveolotomy, nearly 50 per cent of the patients will sustain blood losses in excess of 300 ml.

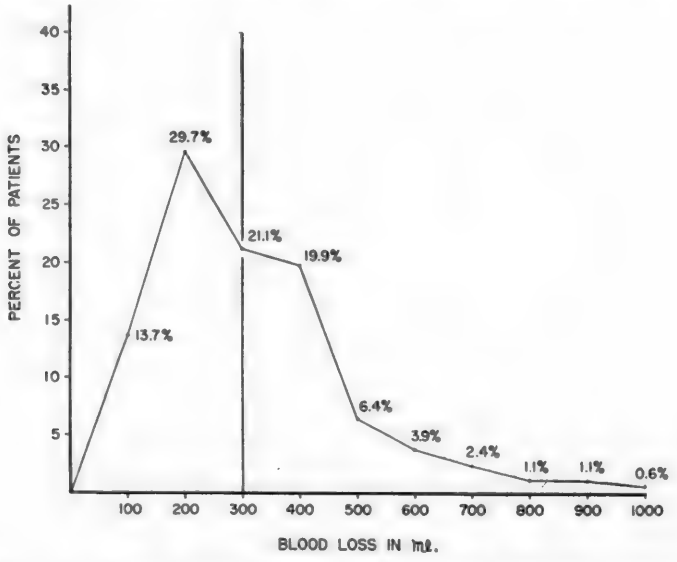

Fig. 3.—Blood loss (in ml.) correlated with number of patients (in percentages).

COMMENT

Prediction of Blood Loss.—In oral surgery, no less than in all types of surgery, it is important that the patient's medical history be obtained and a case "work-up" be made. It is sometimes not an easy matter to obtain a rather thorough medical history from dental patients who, even though hospitalized, rarely can see any connection between the oral cavity and systemic diseases. Since the patient's preoperative general condition and a history of bleeding tendency are of paramount concern to the oral surgeon, the time and effort required to educate these patients to the necessity and validity of obtaining such a history are more than worth while.

The fact that patients were encountered in this series who bled profusely during oral surgery despite having normal blood platelet counts, bleeding, clotting, and prothrombin times on hematologic study might be explained by tissue tone. Hyperemia or the presence of granulation tissue increases the bleeding potential.

The extent of hemorrhage can be influenced slightly by mechanical factors such as suctioning and the use of dry, sterile sponges. Although the use of aspirators and sponges is essential to maintenance of a dry field, this influence

on hemorrhage can be lessened by the use of sterile sponges wrung out of sterile saline solution or water, and by immediate suturing of opened wounds.

Operative Technics.—In an attempt to ascertain whether blood loss would be materially affected by different operative technics, blood loss was compared with the technics employed by the 8 oral surgeons represented in this study. Their extraction technics fell into two major categories: (1) extraction followed by surgical incision and reflection of mucoperiosteal tissues, alveolotomy, curettage, and closure; and (2) surgical incision, reflection of mucoperiosteal tissues and alveolotomy, followed by extraction, curettage, and closure. Seven of the 8 surgeons employed the first technic described. The one employing the second technic is represented by 9 cases, which was considered too small a number for statistical analysis.

Six of the 7 surgeons in the first category employed a broad-field approach, operating on an entire quadrant or more before final closure was made. The remaining surgeon extracted a maximum of 3 or 4 teeth at one operation.

When the case material was studied separately on the basis of these 3 distinctly different operative technics, the number of patients in the higher blood loss brackets increased correspondingly as the duration of surgery increased in each category, the highest losses occurring, as would be expected, in the road-field approach of the second category. When 16 or more teeth were extracted, the average blood loss in this group (51 patients) was 320 ml., with an average of 19 teeth extracted and an average of 45 minutes operating time. The 115 patients represented by the small-field approach sustained an average blood loss of 200 ml., with an average of 17 teeth extracted and an average of 27 minutes operating time. When 28 or more teeth were extracted, the median blood loss of the first group was 715 ml., while in the second group it was 290 ml., a proportionate difference that was sustained when the number of extractions was divided into 6 groups by increments of 5. The 9 patients in the second category (too few for significance) sustained an average blood loss of 188 ml., with an average of 19 teeth extracted in an operation lasting 39 minutes.

While definite conclusions cannot be drawn from the present series, quantitative blood loss apparently is influenced by operating technic. With repeated studies on large series, a table could be evolved from which, to a large extent, individual blood losses could be predicted. Office cases and hospital cases, however, cannot validly be compared. In this series, when less than 16 teeth were extracted, there was little difference between the groups as regards operative technic; it was only when 16 or more teeth were extracted that the differences were striking.

Our primary concern, however, should not be the quantitative loss of blood, per se, but the ability to evaluate and to some extent prognosticate individual blood losses so that replacement therapy may be instituted whenever needed to maintain physiologic fluid balance. With adequate and early replacement therapy available, and with criteria for actual losses established, the operative procedure elected by the individual oral surgeon is of secondary importance since the well-being of the patient will be safeguarded.

Recovery of Patient.—It was consistently found in this study that postoperatively the tissues of the mouth of the patient who had sustained a large blood loss were in better condition than those of the patient who had had only minimal blood loss. This might be explained as due to the flushing effect of the hemorrhage, the function of the reticuloendothelial system, and, chiefly, the presence of a greater quantity of the essential tissue-building elements as afforded by the increased vascular bed in the gingival tissues of these patients. This does not mean that the systemic recovery of the patient who has hemorrhaged profusely occurs with speed. It is general knowledge that the red cell replacements lag from 6 to 8 weeks following blood loss, whereas plasma volume replacements occur within 24 hours.

The effect of reduced blood volume due to whole-blood loss can be just as disastrous to the patient undergoing surgery in the mouth as elsewhere in the body. Oral surgical patients, however, can sustain rather high blood losses and suffer no immediate apparent ill effects. Perhaps we are favored by the size of the vessels involved. The rate of hemorrhage in extraction is rather uniform, with no sudden gushes from large arteries as in general surgery, and a slow steady reduction of blood volume is better tolerated than are fluctuant rates of hemorrhage.[3]

Because the type of hemorrhage characteristic of oral surgery does not have as profound an effect upon the physiologic mechanism does not mean that replacement therapy of some form is not indicated. On the contrary, replacement therapy is definitely indicated, if only for purposes of maintaining fluid levels within the tissue fluid compartment. When whole blood is lost the fluids of the tissue compartment enter the vascular compartment to maintain the blood volume. The amount of fluid entering the vascular system governs the resultant state of dehydration within the tissue compartment, which is further affected by insensible fluid losses. Therefore, to maintain the proper fluid level intravenous administration of a solution of 5 or 10 per cent dextrose in water is indicated in those patients in whom anticipated blood losses will exceed 300 ml. Precautions governing the administration of intravenous fluids should be observed. Intravenous administration is indicated when the discomfort of surgery in the oral cavity makes these patients extremely reluctant to accept fluids by mouth.

The administration of whole blood should be considered when hemorrhage is in excess of 500 ml. When a systemic condition is present, the patient's physician should be consulted. The untoward sequelae resulting from promiscuous use and abuse of blood transfusions should be common knowledge to anyone employing this therapy.

In those cases in which replacement therapy is deemed unnecessary, the postoperative therapeutic regimen should include a high-quality protein diet, vitamin supplements of B complex and C, and iron in the form of ferrous sulfate. The possibility of postextraction hemorrhage of considerable magnitude also must always be borne in mind.

SUMMARY

Blood loss was determined by a gravimetric method, as described, in a series of 175 patients admitted to the Millard Fillmore Hospital by 8 oral surgeons for extraction with alveolotomy under general anesthesia. Blood loss was found to compare with that incurred in many major general surgical operations as culled from the literature (*Table II*). Average losses exceeded those for simple mastectomies, inguinal herniorrhaphies, and hemorrhoidectomies, and maximum losses were in excess of those for hysterectomies and other pelvic operations, nephrectomies, cholecystectomies, herniorrhaphies (all types), and thyroidectomies.

TABLE II
BLOOD LOSS IN VARIOUS OPERATIONS (AMERICAN LITERATURE)[2]

TYPE OF OPERATION	NUMBER OF CASES	MAXIMUM LOSS (C.C.)	MINIMUM LOSS (C.C.)	AVERAGE LOSS (C.C.)
Appendectomies	110	62	4	10
Cholecystectomies	26	357	51	171
Herniorrhaphies				
Inguinal	40	263	6	58
Ventral	4	306	48	146
Thyroidectomies	43	888	16	249
Hemorrhoidectomies	20	150	20	55
Transurethral prostatic resection	237	1,254	4	296
Pelvic surgery				
Hysterectomies	43	629	40	358
Others	34	579	14	153
Brain operations	19	2,150	574	1,287
Gastric resections	99	1,432	150	441
Mastectomies				
Simple	3	220	190	205
Radical	27	1,272	196	690
Nephrectomies	12	845	208	484
This study (total hospital experience)	175	912	35	223

CONCLUSIONS

1. Quantitative determination of blood losses sustained in oral surgery in a series of 175 patients representing 8 oral surgeons showed losses ranging from 35 to 912 ml., with an average loss of 223 ml. with extraction of 18 teeth and alveolotomy in an operative procedure lasting 30 minutes.

2. The extent of hemorrhage in oral surgery has been vastly underestimated. The blood loss associated with extraction of teeth and alveolotomy in the average patient compares with blood losses encountered in major surgery. It is recommended that blood loss determinations be made a routine procedure during exodontia.

3. Some correlation between blood loss and age and sex of the patient might have been expected. We were unable to demonstrate any in this series.

4. Time and blood loss are direct surgical antagonists. The longer the operating time, the greater the blood loss.

5. Blood losses in oral surgery may be predicted to some extent. A thorough medical history and preoperative complete blood count on all patients,

as well as the condition of the tissues of the mouth, afford valuable information. In average patients, blood losses may be anticipated in conformity with individual operating time and technic.

6. While having some guide to prognosing blood loss is valuable, in cases of severe blood loss a method of calculating such loss as it occurs is invaluable.

7. Although the rate of hemorrhage in oral surgery seems to have a less profound immediate physiologic effect than in other types of surgery, any appreciable reduction of blood volume can be just as disastrous to the patient.

I wish to express my sincere appreciation to Dr. G. G. Pritchard, chairman of the Oral Surgery Department; to the Oral Surgery Staff; to the Department of Anesthesiology; and to the Section of Hematology for their cooperation in making this study possible.

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THE EFFECT OF ARTIFICIAL WATER FLUORIDATION ON THE SOLUBILITY OF TOOTH ENAMEL

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STUDIES HAVE shown that tooth enamel exposed in vitro to a fluoride solution has a reduced solubility in acids.[1-3] It has been demonstrated, also, that enamel from natural fluoride areas has a greater resistance to acid decalcification than does enamel from fluoride-free areas.[4] It would be of interest to determine if enamel from an area of artificial water fluoridation displays a similar resistance to acids as does enamel from natural fluoride areas.

This study was designed to determine the acid solubility of deciduous enamel from children born prior to and at least 6 months after artificial fluoridation of a communal water supply and to compare this with the solubility of enamel from a fluoride free area.

For this study, caries free, naturally exfoliated anterior deciduous teeth were utilized from the following groups of children, whose complete histories were known:

1. From Newburgh, New York, children whose teeth had erupted prior to fluoridation, but exposed to the water (1 ppm fluorine) posteruptively for 5 years.

2. From Newburgh, New York, children born during artificial water fluoridation (1 to 1.2 ppm fluorine).

3. From Rochester, New York, children then drinking relatively fluorine-free water.

PROCEDURE

All exfoliated teeth were cleaned and pulverized to pass through a 60-mesh screen. The enamel from each of the groups of teeth were pooled, although several of the teeth from the postfluoride group were considered individually. The enamel was separated from the remainder of the tooth substance by the flotation method of Manly and Hodge.[5]

Acid solubilities of the enamel samples were determined by a modification of the method employed by Volker.[6] This variation in technic avoids the necessity of transferring the sample from one container to another, thus eliminating one possible source of error. Fifty milligram samples of enamel, previously dried for 1 hour in an oven, then desiccated for 1 hour, were

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weighed out in a previously tared Buchner filter funnel. The outlet of the funnel was placed into a rubber hose connected to a source of compressed air and the funnel and tube unit placed in a constant temperature water bath of 37° C. A gentle stream of air was permitted to flow through the funnel.

To the 50 mg. sample of enamel was added 20 c.c. of a 0.2 M HAc/NaAc buffer solution of pH 4. A cover glass was placed over the funnel and the stream of air so regulated that there was sufficient agitation to keep the enamel particles in a constant state of motion in the solution and prevent any of the buffer solution from escaping from the funnel through the rubber tubing. The reaction was continued for 1 hour. The funnel was then removed from the tubing and the water bath and placed on a suction flask. The cover glass and funnel were washed 3 times with distilled water. The funnel and contents were dried in an oven for 1 hour and allowed to cool to room temperature in a desiccator for 1 hour. The funnel and contents were then weighed. The difference between the original weight of the funnel, subtracted from the final weight of the funnel and contents gave the weight of the material remaining and the amount of enamel dissolved. Samples of enamel were run in groups of 4.

DATA

Ten analyses were run on the pooled enamel of teeth of the Newburgh children erupted prior to fluoridation. Table I presents these findings. It will be observed that the average enamel remaining after 1 hour of contact with the acetic acid–sodium acetate buffer was 36.6 mg.

TABLE I
THE SOLUBILITY OF DECIDUOUS ENAMEL FROM POOLED SAMPLES OF TEETH
(1943—PREFLUORIDATION)

ANALYSIS NUMBER	INITIAL WEIGHT (MG.)	WEIGHT UNDISSOLVED (MG.)	DEVIATION FROM AVERAGE (MG.)
1	50.0	35.9	0.7
2	50.0	36.6	0.0
3	50.0	34.7	1.9
4	50.0	37.0	0.4
5	50.0	37.6	1.0
6	50.0	36.1	0.5
7	50.0	36.8	0.2
8	50.0	37.7	1.1
9	50.0	36.9	0.3
10	50.0	37.2	0.6
Average		36.6	

The findings of 6 analyses run on the pooled and individual tooth samples of enamel from Newburgh children born at least 6 months after fluoridation had began is presented in Table II. The average weight of enamel undissolved was 38.6 mg.

The solubility of pooled samples of Rochester enamel (fluorine free) is given in Table III. The average weight of deciduous enamel undissolved for this group was 36.1 mg.

Table IV presents a summary of the data from the 3 groups.

TABLE II
THE SOLUBILITY OF DECIDUOUS ENAMEL FROM POOLED SAMPLES AND SINGLE SAMPLES OF TEETH (1945—POSTFLUORIDATION)

ANALYSIS NUMBER	INITIAL WEIGHT (MG.)	WEIGHT UNDISSOLVED (MG.)	DEVIATION FROM AVERAGE (MG.)
1	50.0	38.7	0.2
2	50.0	39.1	0.6
3	50.0	37.7	0.8
4	50.0	39.0	0.5
5	50.0	38.5	0.0
Average		38.6	

TABLE III
THE SOLUBILITY OF DECIDUOUS ENAMEL FROM POOLED SAMPLES OF ROCHESTER TEETH—DENTAL DISPENSARY

ANALYSIS NUMBER	INITIAL WEIGHT (MG.)	WEIGHT UNDISSOLVED (MG.)	DEVIATION FROM AVERAGE (MG.)
1	50.0	34.1	2.0
2	50.0	36.4	0.3
3	50.0	35.4	0.7
4	50.0	35.9	0.2
5	50.0	36.7	0.6
6	50.0	35.7	0.4
7	50.0	36.3	0.2
8	50.0	36.8	0.7
9	50.0	36.1	0.0
10	50.0	35.7	0.4
11	50.0	35.9	0.2
12	50.0	37.3	1.2
13	50.0	36.1	0.0
14	50.0	36.8	0.7
15	50.0	36.5	0.4
Average		36.1	

TABLE IV
A SUMMARY OF THE DATA ON SOLUBILITY OF ENAMEL FROM FLUORIDATED AND NON-FLUORIDATED COMMUNITIES

	NUMBER OF SAMPLES	MEANS	STANDARD DEVIATIONS
Enamel (Newburgh—prefluoridation)	10	36.65	0.89
Enamel (Newburgh—postfluoridation)	5	38.60	0.51
Enamel (Rochester—fluorine free water)	15	36.12	0.73

DISCUSSION

There is a statistically significant difference, when the T test is applied to the means, between the solubility of the Newburgh enamel produced after fluoridation of the drinking water as compared to the enamel produced prior to fluoridation and of Rochester, New York, enamel. This mechanism might account for the resistance to dental caries observed in artificial fluoride areas.

Although the difference between the solubility of the Newburgh enamel from teeth erupted prior to fluoridation is not significantly different from that of Rochester enamel, nevertheless, the slight reduced solubility observed in the prefluoridation Newburgh enamel might have important implications. It may indicate a topical effect of important magnitude. Since, the outer layers of enamel are richer in fluorine, the slight reduced solubility of the

entire prefluoride enamel might dilute and mask a rather marked reduced solubility of the outer layer of enamel.[7] This would aid in explaining the posteruptive effect of fluorine on teeth observed in animals by McClure[8] and observed in humans by Detherage.[9] One might speculate from this data that the posteruptive maturation of teeth, observed clinically in humans and experimentally in animals, might be due to a great extent to this type of phenomenon. Should this hypothesis prove to be valid, it would mitigate against the value of any fluoride tablets swallowed without being dissolved in the mouth or ingested only periodically. It would appear that the most effective method of systemic fluoride administration would be in a fluid medium, brought in contact with the surfaces of the teeth at frequent intervals. The observations of Soggnnaes and Armstrong,[10] that the anterior teeth from a fluoride area have a greater fluorine content than the posterior teeth, lend credence to this hypothesis. Water fluoridation offers the most effective means of using fluorine for caries prevention, for by so doing, not only is the fluoride ingested during tooth formation, but bathes the tooth surfaces after eruption as well.

CONCLUSIONS

1. Deciduous enamel from an area of artificial water fluoridation has a reduced solubility in acid as compared to enamel from fluoride-free areas.

2. Enamel exposed to fluoridated water after eruption shows a very slight reduction in acid solubility as compared to enamel from fluoride-free areas.

3. Artificial water fluoridation appears to be the best means of utilizing fluorine for dental caries reduction.

The authors wish to express their thanks to Drs. H. C. Hodge, D. B. Ast, and J. F. Volker for their many helpful suggestions.

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INVESTIGATIONS OF THE ANTI-ENZYMATIC ACTION OF FLUORIDE AT THE ENAMEL SURFACE

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INTRODUCTION

ONE of the mechanisms of the action of fluoride that has been considered in the prevention of dental caries is its function as an enzyme inhibitor. Borei[1] has reviewed extensively the role of fluoride as an inhibitor of cellular oxidation and it is apparent that the concentrations of fluoride necessary to inhibit glycolysis or oxidation (19 to 190 ppm fluoride ion) in intact cells are far greater than the concentrations found in saliva by McClure.[2] Notwithstanding these biochemical observations, reports have occurred in the literature which claim that low concentrations of fluoride ion (0.5-1.0 ppm) may affect acid formation by oral organisms. This has not been confirmed by direct observation of acid formation by mixed suspensions of oral organisms (Lilienthal[3]). Furthermore it has been suggested that fluorosed enamel may prevent acid formation in pure cultures of several oral bacteria (Bibby and Van Kesteren[4]). These latter observations have not been confirmed. Owing to the fact that the mechanism of fluoride inhibition of dental caries has not been clarified, and as a result of the studies on acid production by salivary sediment, it is necessary to re-evaluate the significance of the factors relating to fluoride ion inhibition of acid formation at the tooth surface.

MATERIALS

Powdered human enamel was prepared free from dentin by the flotation method of Manly and Hodge.[5] The synthetic hydroxyapatite used was $Ca_3(PO_4)_2.2H_2O$. This compound and the powdered enamel both gave identical x-ray diffraction spectrograms, using a Geiger x-ray spectrometer. The sample of naturally occurring fluorapatite used was from Oregon, Canada.

Human tooth crowns were selected and the exposed dentin and cementum surfaces were sealed with an inert resinous mixture consisting of 80 Gm. resin in 20 Gm. lanolin.

METHODS

Treatment of Teeth and Powdered Enamel With NaF.—The material to be treated was shaken in a water-bath at 38° C. for 30 minutes with 3.0 ml. 2 per cent NaF. At the end of this time the suspended material was centrifuged

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down and the supernatant fluid discarded. The material was washed with 10 ml. quantities of distilled water and the fluoride concentration of the washings was determined by the method of Megregian and Maier.[6] The volumes in the estimations used were reduced to one-tenth of those recommended and the color which developed was measured in 2.0 cm. diameter tubes in a Unicam diffraction grating spectrophotometer (Model SP350).

The Determination of Rates of Acid Formation.—Conventional manometric methods were adopted (Umbreit, Burris, and Stauffer[7]) with pooled salivary sediment suspended in bicarbonate giving a final pH of 6.8 with the gas phase 5.5 per cent CO_2 and 94.5 per cent N_2.

RESULTS

Acid Formation in the Presence of Powdered Enamel, Synthetic Hydroxyapatite and Fluorapatite.—Powdered human enamel, synthetic hydroxyapatite and natural fluorapatite, when added in 50 mg. quantities to salivary sediment, gave identical rates of acid formation from suerose. In addition, powdered enamel and hydroxyapatite, treated with NaF for 30 minutes and washed once with 10 ml. distilled water, both inhibited the rate of acid formation. The degree of inhibition was similar to that produced by 50 mg. CaF_2 (Fig. 1). An excess of these materials was used in the experiments in order to provide ionic conditions similar to those which would exist at the tooth surface.

The Effect of Enamel and Hydroxyapatite, Both Exposed to Fluoride.—Further investigations were made of the inhibitory effect of artificially fluorosed enamel and synthetic hydroxyapatite. Samples of each (50 mg.) were treated with 2 per cent NaF and, after washing with water from one to four times, added to the acid-producing system. Analysis of the washings showed a decrease in fluoride content from the first to the fourth washing. However, the powdered enamel showed a greater inhibitory action on acid production. It must be concluded that the fluoride ion concentration was not solely responsible for these results as a decrease, not an increase, in fluoride in the washings was noted. Unwashed, normal, untreated powdered enamel did not have a significant effect on the rate of acid formation (Fig. 2). Fluoride-treated hydroxyapatite also markedly inhibited acid production but the increased inhibition produced by washing was not observed (Fig. 2). Untreated hydroxyapatite only slightly affected the rate. Table I shows the fluoride values found in the washings of the enamel and hydroxyapatite used in these experiments.

TABLE I

FLUORIDE CONCENTRATION* (PPM) IN WASHINGS OF ENAMEL AND HYDROXYAPATITE, BOTH EXPOSED TO FLUORIDE

	ENAMEL SAMPLE					CORRESPONDING CURVE IN FIG. 2	HYDROXYAPATITE SAMPLE					CORRESPONDING CURVE IN FIG. 2
	1	2	3	4	MEAN		1	2	3	4	MEAN	
First washing	432	432	400	280	386	–	760	720	640	840	740	–
Second washing	–	32	22	28	27	a	–	80	60	60	67	a_1
Third washing	–	–	22	20	21	b	–	–	40	60	50	b_1
Fourth washing	–	–	–	20	20	c	–	–	–	48	48	c_1

*Concentrations expressed for a volume of 2.5 ml.

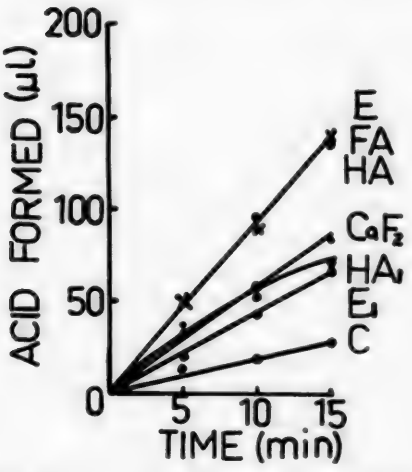

Fig. 1.—The effect of enamel, hydroxyapatite and fluorapatite on acid formation.

System: 2.0 ml. salivary sediment (6 mg. dry wt.), 1×10^{-3} M sucrose in 4×10^{-3} M $NaHCO_3$. Volume made up to 2.6 ml. with 4×10^{-3} M $NaHCO_3$.

Additions: *E* = 50 mg. powdered enamel, *FA* = 50 mg. fluorapatite, *HA* = 50 mg. hydroxyapatite, CaF_2 = 50 mg. calcium fluoride, *HAI* = 50 mg. fluorosed hydroxyapatite, *EI* = 50 mg. fluorosed enamel, *C* = control, without sucrose.

Rate of acid formation with sucrose only was the same as shown for *E*, *FA*, and *HA*.

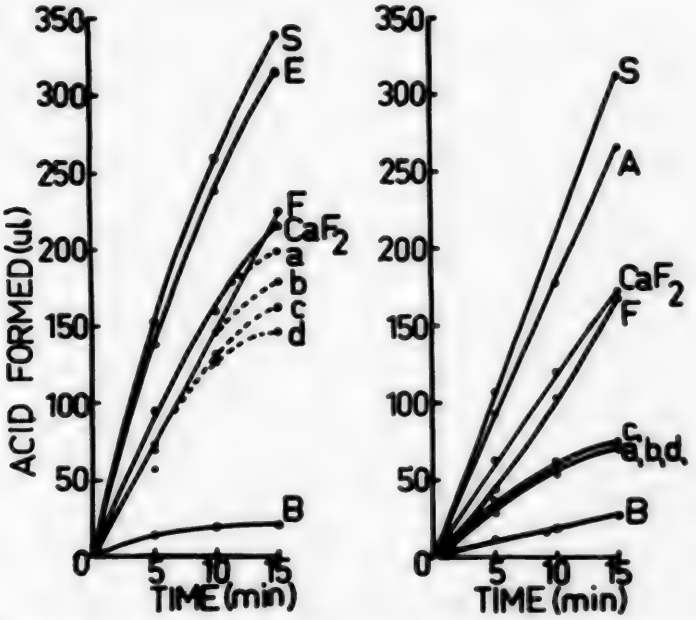

Fig. 2.—Inhibition of acid formation by fluorosed enamel and hydroxyapatite.

System: 2.0 ml. salivary sediment (13 mg. dry wt.) and 1×10^{-2} M sucrose in 4×10^{-3} M $NaHCO_3$. Volume made up to 2.6 ml. with 1×10^{-3} M $NaHCO_3$.

Additions: *E* = unwashed powdered enamel (50 mg.), CaF_2 = calcium fluoride (50 mg.), *F* = 32 ppm fluoride ion, *a,b,c,d* = 50 mg. fluorosed enamel washed 1×, 2×, 3× and 4× with 10 ml. distilled water, *B* = blank, without sucrose, *S* = control for sucrose, without other additions, *A* = synthetic hydroxyapatite (50 mg.), a_1,b_1,c_1,d_1 = fluorosed hydroxyapatite washed 1×, 2×, 3× and 4×, respectively, as for enamel.

The fluoride values shown in this table were calculated from the concentrations found in the 10 ml. quantities of distilled water used to wash the samples of enamel and hydroxyapatite. In order to gain some idea of the concentration produced by the addition of the fluorosed enamel or hydroxyapatite in the system studied, these concentrations were corrected to represent those for 2.5 ml., which was the volume of fluid in the manometers. Further, the fluoride concentration produced by a sample of enamel or hydroxyapatite washed once and then suspended in 2.5 ml. fluid would correspond to the values obtained from the second washing of the enamel, not the first. The fluoride concentration produced by a sample of enamel washed n times would correspond to the values found in $(n + 1)$th washing. It should be noted that the concentrations found for fluorosed hydroxyapatite and enamel exceed 8 ppm fluoride: the hydroxyapatite samples adsorbed considerably more fluoride than the enamel. The latter was of a larger particle size than the former, which could readily explain this observation. In each instance the high values found in the first washing were most probably due to carry-over of a small amount of the 2 per cent solution of sodium fluoride. The solution of fluoride from the treated enamel and hydroxyapatite appears to have been independent of the solution of calcium because fluoride concentrations greater than 8 ppm cannot be attained in the presence of abundant calcium ions owing to the insolubility of CaF_2. On repeating the experiment with powdered enamel and estimating the fluoride content of the washing fluids and also of the fluid in the manometer cups at the end of the experiment, slightly higher values were found for fluoride than those calculated from the washings (Table II). The effect of these samples of fluorosed enamel on acid formation is shown in Fig. 3, where it can be seen that the greater the number of times the NaF-treated enamel was washed, the greater was the inhibition. The sample of enamel treated with water instead of with 2 per cent NaF and washed once also slightly inhibited acid production.

TABLE II

A COMPARISON OF FLUORIDE CONCENTRATIONS IN WASHINGS OF FLUOROSED ENAMEL WITH THOSE PRODUCED BY THE ENAMEL IN THE FERMENTATION SYSTEM

	FLUORIDE (PPM) IN WASHINGS OF ENAMEL SAMPLES						FLUORIDE (PPM) FOUND IN MANOMETERS
	1	2	3	4	5	MEAN	
First washing	240	360	320	280	420	324	–
Second washing	–	32	24	28	22	26	33
Third washing	–	–	22	24	20	22	25
Fourth washing	–	–	–	22	12	17	21
Fifth washing	–	–	–	–	12	12	18

When enamel treated with distilled water instead of with 2 per cent NaF was added to salivary sediment, the results shown in Fig. 4 were obtained. These show that enamel treated with distilled water and washed several times also produced a marked inhibition of acid formation, as did enamel treated with 2 per cent NaF. Further the effect of 50 mg. calcium fluoride was as

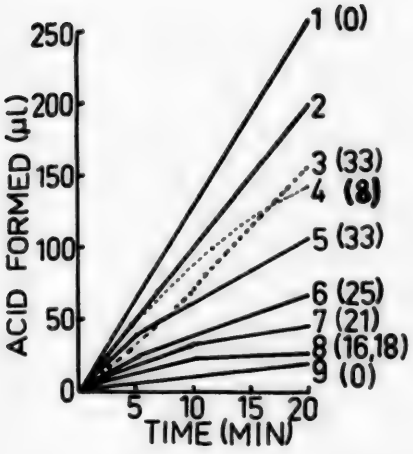

Fig. 3.

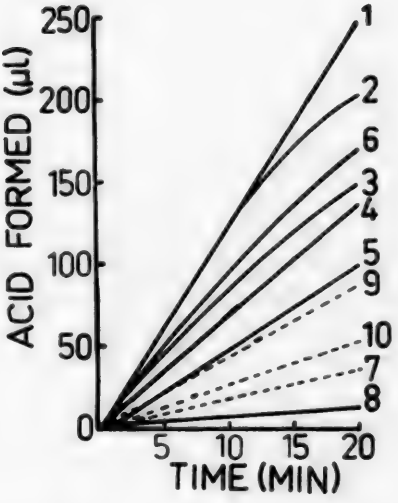

Fig. 4.

Fig. 3.—Inhibitory effect of washed fluorosed enamel and fluoride content of the system.

System: 2.0 ml. salivary sediment (6 mg. dry wt.) and 1×10^{-2} M sucrose in 4×10^{-3} M $NaHCO_3$, 4×10^{-3} M $NaHCO_3$ and other additions to 2.6 ml.

Curve 1, 1×10^{-2} M sucrose only; Curve 2, enamel (50 mg.) treated with H_2O and washed once; Curve 3, 32 ppm fluoride ion; Curve 4, CaF_2 (50 mg.); Curve 5, fluorosed enamel, washed once; Curve 6, fluorosed enamel, washed twice; Curve 7, fluorosed enamel, washed three times; Curve 8, fluorosed enamel, washer four and five times; Curve 9, blank, without added sucrose. Values in parenthesis are the fluoride concentrations (ppm) found at the end of the experiment.

Fig. 4.—A comparison of the inhibitory effects of washed, fluorosed and normal enamel.

System: 2.0 ml. salivary sediment (6 mg. dry wt.), 1×10^{-2} M sucrose in 4×10^{-3} M $NaHCO_3$. Volume made up to 2.6 ml. with 4×10^{-3} M $NaHCO_3$.

Curve 1, sucrose only; Curve 2, CaF_2 (25 mg.); Curve 3, CaF_2 (50 and 100 mg.); Curve 4, fluoride ion (32 ppm); Curve 5, fluoride ion (64 ppm); Curve 6, enamel treated with water and washed once; Curve 7, enamel treated with water and washed three times; Curve 8, enamel treated with water and washed five times; Curve 9, fluorosed enamel washed once; Curve 10, fluorosed enamel washed three times; Curve 11 (as Curve 7): fluorosed enamel washed five times; Curve 12 (as Curve 7): control without added sucrose.

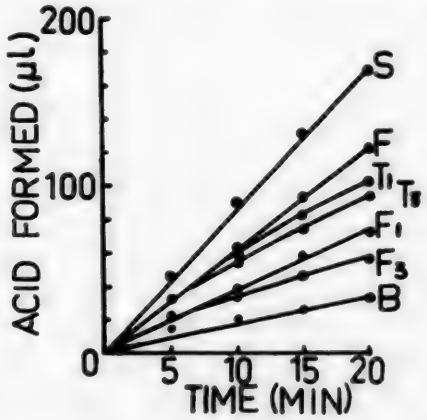

Fig. 5.—Acid formation by sediment in the presence of fluorosed and normal tooth crowns.

System: 2.0 ml. salivary sediment (4 mg. dry wt.) and 1×10^{-2} M sucrose in 4×10^{-3} M $NaHCO_3$. Volume made up to 2.6 ml. with 4×10^{-3} M $NaHCO_3$.

Curve S, sucrose only; Curve F, fluoride ion (32 ppm); Curve T1 and T3, tooth crowns (4) incubated with distilled water and washed once and 3 times with distilled water; Curve F1 and F3, fluorosed tooth crowns washed once and three times; Curve B, no added sucrose.

great as that of 100 mg. In the presence of 25 mg. CaF_2, sufficient to give the maximum fluoride concentration of 8 ppm F^-, the rate of acid formation was not inhibited during the first 10 minute period but was in the second.

The Effect of NaF-Treated Teeth.—The inhibitory effect produced by repeated washing of powdered enamel with distilled water may have been associated with the size of the enamel particles. When tooth crowns were used, however, the enhanced effect caused by washing was again found (Fig. 5) and the fluoride-treated crowns were more inhibitory than those treated only with water.

DISCUSSION

The results reported in this paper do not support an anti-enzymatic effect of fluoride as a mechanism of inhibition of dental caries. Any such action of fluoride in the mouth is determined by the fluoride ion concentration. The maximum concentration found by McClure[2] in saliva was 0.1 ppm fluoride, which has been shown to be without effect on acid formation (Lilienthal[3]). A remaining possibility is an increased local concentration of fluoride at the tooth surface, due to a solution of the enamel and/or adsorbed fluoride. If the mineral phase of enamel consisted only of fluorapatite it would contain 3.67 per cent fluoride (McConnell[8]). However, fluorapatite was found to have no effect on the rate of acid formation by salivary sediment. Although the fluoride content of fluorapatite is relatively high, it is not readily available as free fluoride and so is unable to inhibit acid formation. Fluoride may also be present on the enamel surface as calcium fluoride (Scott, Picard and Wyckoff[9]). It has been shown that CaF_2 in greater amounts than its solubility in the volume used produced a slight inhibitory effect on acid formation by the suspension of mixed oral organisms. On increasing the amount of solid CaF_2 from 25 to 50 mg. this inhibition was increased, which suggests an effect caused by factor(s) other than fluoride concentration.

Artificially fluorosed powdered enamel and synthetic hydroxyapatite gave a similar inhibition of acid formation. The values for the fluoride concentration produced by the solution of fluorosed enamel and hydroxyapatite in addition to CaF_2 are each limited in the presence of excess Ca^{++} by the solubility of CaF_2 (16 ppm). These values are much greater than can be produced on the tooth surface by the solution of enamel substance containing up to 0.1 per cent fluoride. Assuming the solubility of tooth substance is equivalent to that of hydroxyapatite (23 ppm), the fluoride liberated would attain a concentration of only 0.02 ppm. The real significance of the observed inhibition of acid formation by fluorosed enamel is influenced, however, by the fact that when enamel was washed with distilled water it produced a marked inhibition of acid production, similar to that of enamel treated with 2 per cent sodium fluoride. Volker, Hodge, Wilson, and Van Voorhis[10] found that the maximum amount of fluoride adsorbed onto a tooth crown was approximately 0.02 mg. In the experimental conditions used in the above investigations, adsorption of

0.02 mg. fluoride per tooth crown would result in a final concentration of 32 ppm F^-, assuming complete solution of the adsorbed fluoride (4 tooth crowns in 2.5 ml. water).

The results obtained with tooth crowns treated with 2 per cent NaF are probably due to solution of the CaF_2 formed or adsorbed fluoride, together with the effect of the enamel exposed by this solution. In the presence of calcium and phosphate concentrations found in saliva, 38 ppm F^- has no effect on acid production (Lilienthal[3]). This concentration (38 ppm F^-) is more than 4 times as great as the maximum attainable (8 ppm) in the presence of excess calcium ions. Not only in saliva which is a supersaturated solution of calcium phosphate, but on the tooth surface, where acid solution of the surface apatite would liberate calcium in addition to fluoride, the concentration of calcium would be sufficient to exceed the 1 to 4 calcium-fluoride ratio to prevent any anti-enzymatic action of fluoride.

The enhanced inhibitory effect observed on washing with water requires further study and among the factors to be considered is the differential solution of adsorbed ions from the surface of the apatite structure.

Considering the very small amount of fluoride bound on the enamel surface, the limitations imposed by the solubility of calcium fluoride and the protective action of calcium ions, the mechanism of the prevention of dental caries with fluoride cannot reasonably be explained by an anti-enzymatic effect on acid production at the tooth surface.

SUMMARY

1. Powdered enamel, synthetic hydroxyapatite and naturally occurring fluorapatite had no effect on the rate of acid formation from sucrose by salivary sediment.

2. Artificially fluorosed, powdered enamel, and synthetic hydroxyapatite inhibited acid production to a similar degree to that caused by calcium fluoride.

3. Washing powdered enamel and fluorosed enamel with distilled water increased their inhibitory action on acid formation. This effect could not be explained by the fluoride ion concentration in the system.

4. A similar increase in inhibition of acid formation on washing was found with either fluorosed or untreated tooth crowns.

5. These observations are discussed in relation to the possible fluoride content of enamel and its solubility. It is concluded that fluoride, in a concentration expected at the tooth surface, does not prevent acid formation.

We are indebted to Mr. A. Schwartz, Department of Physics, New South Wales University of Technology, for the x-ray diffraction analyses of enamel and hydroxyapatite.

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THE EFFECT OF FLUORIDE ON ACID FORMATION BY SALIVARY SEDIMENT

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INTRODUCTION

THE action of fluorides in the prevention of dental caries is due to the effects of the fluoride ion. Its mode of action is thought to be due to either or both of two mechanisms. The first is the production of dental enamel more resistant to solution in acids by the action of fluoride ion during calcification and following tooth eruption. The second effect of fluoride ion is the inhibition of acid formation by microorganisms at the tooth surface—an anti-enzymatic action.

Wright and Jenkins[1] claimed that a fluoride concentration as low as 0.5 ppm will significantly inhibit acid production and prevent changes of pH in saliva-glucose mixtures incubated at 37° for 4 hours and 24 hours. Using pure cultures of several oral organisms, Bibby and Van Kesteren[2] claimed that less than 1 ppm fluoride ion inhibited acid production during growth. From a comparison of curves for growth and for acid formation in the results of the latter workers it is apparent that fluoride had its effect only when the cell numbers had reached a maximum and the organisms were already in the decline phase of growth. During logarithmic growth even 100 ppm sodium fluoride (i.e., 45 ppm fluoride ion) had no effect on acid formation by oral strains of lactobacilli or streptococci. Cox and Levin[3] claimed further that, in media containing only 0.1 ppm fluorine, acid production was "remarkably inhibited."

In order to reinvestigate this problem of the anti-enzyme action of the fluoride ion on oral microorganisms, salivary sediment was used as a source of oral bacteria. This material is a sample of the mixed bacterial population of the mouth and thus approaches more closely the flora which may be found on tooth surfaces.

MATERIALS AND METHODS

Paraffin activated saliva was collected and centrifuged for 10 minutes at 3,000 rpm. The deposit was used unwashed or washed with distilled water as indicated. Owing to the possibility of the presence of reducing sugar in saliva collected after a meal (Lundquist[4]), the accompanying high values for acid production without added carbohydrate were avoided by collecting the saliva approximately 2 hours after the previous meal. When samples of saliva were

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collected within 1 hour of the midday meal, acid formation by the sediment was appreciable (50 μl/20 min.) in the absence of added carbohydrate, because of the degradation of stored carbohydrate within the cells.

Estimation of the Rates of Acid Formation.—Conventional manometric methods (Umbreit, Burris, and Stauffer[5]) were employed using a mixture of 5.5 per cent CO_2 and 94.5 per cent N_2 in the gas phase. A bicarbonate concentration was chosen to give pH 6.8 in the system and it was used for the suspension of the salivary sediment and for the preparation of the solutions. The quantity of cells used differed from one experiment to another but was between 5 and 10 mg. dry weight per manometer. Because CO_2 is not formed in significant amounts from sucrose by salivary sediment (Lilienthal[6]), the actual gas output was recorded as acid produced. This was also done when glucose was the substrate, although approximately 10 per cent of these values may have been CO_2 and not acid.

RESULTS

Preincubation of Salivary Sediment with Fluoride.—When fluoride (19 ppm) was incubated at 38° for 30 minutes with salivary sediment before addition of sucrose, the rate of acid formed from sucrose was the same as that for sediment incubated for the same period in the absence of fluoride. In this experiment, the fluoride concentration (1×10^{-3} M, 19 ppm) had a slight effect on the rate of acid formation from sucrose (Fig. 1).

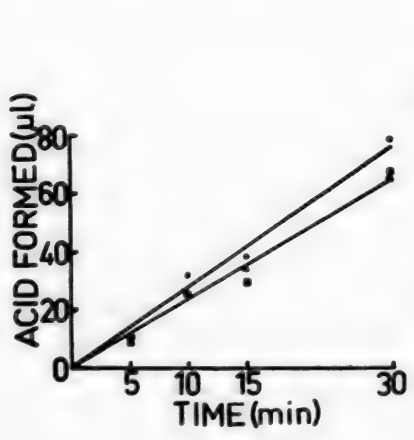

Fig. 1.

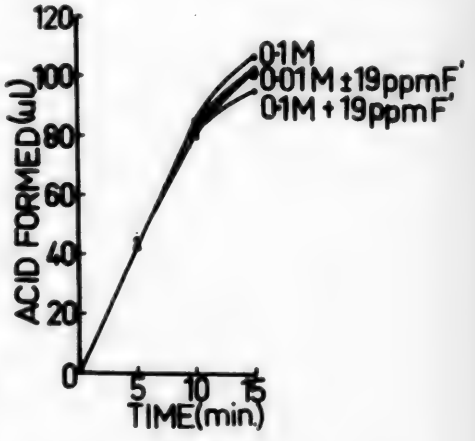

Fig. 2.

Fig. 1.—Preincubation of sediment with fluoride and acid formation.

System: salivary sediment (2.0 ml.) in 4×10^{-3} M $NaHCO_3$, 0.2 ml. sucrose (0.1 M), 0.25 ml. NaF (0.01 M) and 4×10^{-3} M $NaHCO_3$ to 2.5 ml. ●——● sucrose only; ▲——▲ 1×10^{-3} M NaF (i.e., 19 ppm) incubated with the cells 30 minutes before addition of sucrose; ■——■ 1×10^{-3} M NaF added with sucrose to the salivary sediment after 30 minutes incubation.

Fig. 2.—Effect of sucrose concentration on rate of acid formation in the presence of fluoride.

System: salivary sediment (2.0 ml.) suspended in 4×10^{-3} M $NaHCO_3$ with additions of sucrose and NaF, also in 4×10^{-3} $NaHCO_3$. Volume made up to 2.5 ml. with $NaHCO_3$.

Because there was no difference between the rates of acid production of fluoride added with the cells or with sucrose, the length of the preincubation period has no significant effect on the fluoride action. In the experiments which follow fluoride was added always to the cells and the sucrose from the manometer side-arm at the commencement of readings.

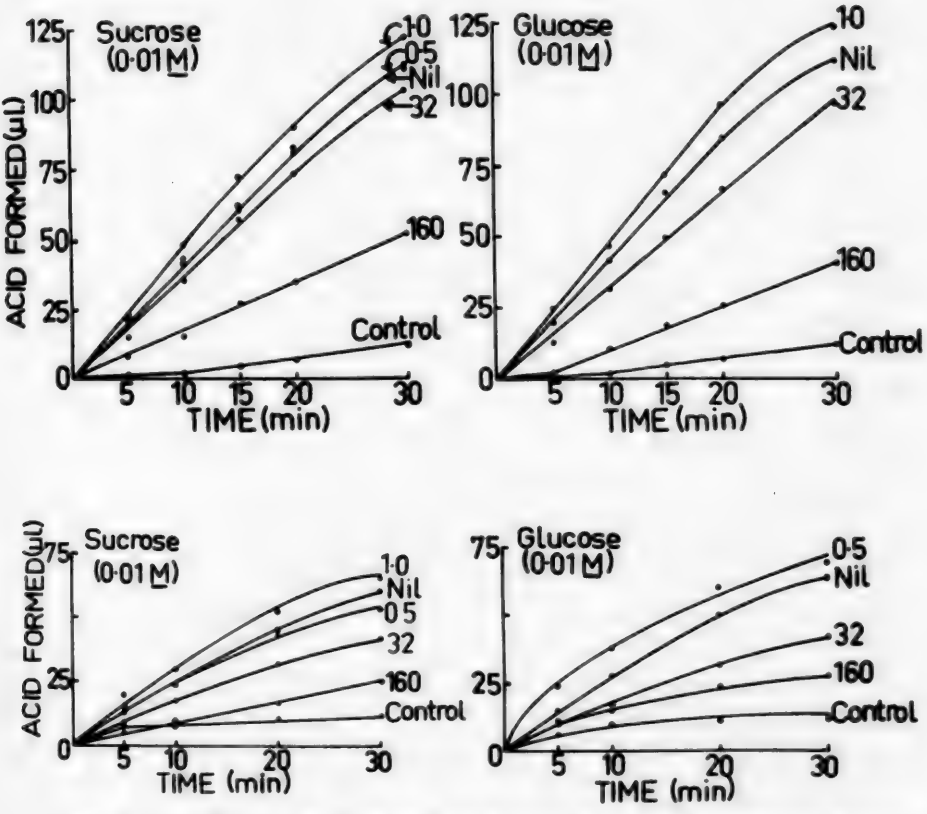

Fig. 3.—Effect of fluoride on the rate of acid formation by salivary sediment from sucrose and glucose.

System: 2.0 ml. sediment in 4 × 10^{-3} M $NaHCO_3$, 0.2 ml. 0.1 M sucrose or glucose and fluoride to give the concentrations indicated. Numbers refer to the final fluoride concentration in the system in ppm. Control cups contained no addition of substrate.

Effect of Substrate Concentration.—With a tenfold difference in sucrose concentration (10 × 10^{-2} M and 1 × 10^{-2} M), the rate of acid formation was identical. Addition of 1 × 10^{-3} M fluoride ion (19 ppm) to each concentration of substrate did not significantly alter the initial rates at which acid was formed (Fig. 2). In this system with salivary sediment 1 × 10^{-2} M sucrose was sufficient for maximum acid formation and was used in the experiments which follow.

The Effect of Fluoride Ion Concentration on Acid Production.—(a) With sediment from pooled saliva: In order to test the effect of a series of concentrations of fluoride on acid formation, saliva obtained from several individuals was

pooled. The sediment from this mixed saliva was sufficient to enable a series of fluoride concentrations to be tested with 1×10^{-2} M sucrose and 1×10^{-2} M glucose as substrates. This was done on 4 separate occasions, the results being shown in Fig. 3. Fluoride ion concentration of 0.5 and 1.0 ppm did not inhibit the production of acid from either of these 2 substrates. On the contrary, a slight stimulation was found. With additions of 32 ppm fluoride ion, acid formation was affected with one exception. The degree of inhibition after 20 minutes was 10 to 30 per cent. Marked inhibition (approximately 50 per cent) occurred in the presence of 160 ppm of fluoride ion.

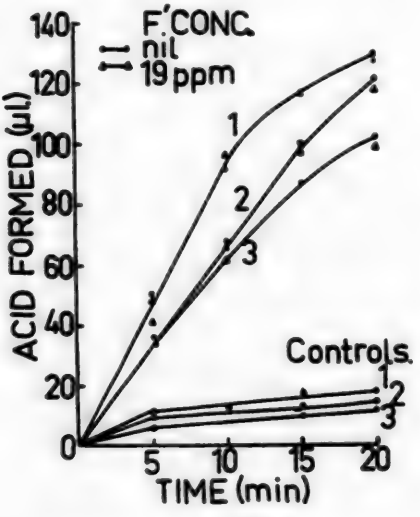

Fig. 4.

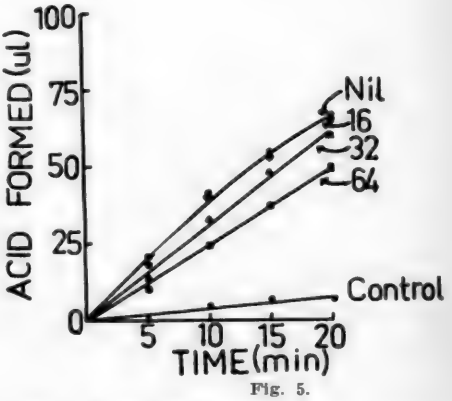

Fig. 5.

Fig. 4.—Effect of 1×10^{-2} M fluoride (19 ppm) on acid formation.

System: salivary sediment, 1×10^{-2} M sucrose and fluoride in 4×10^{-2} M $NaHCO_3$ and volume made up to 2.5 ml. with $NaHCO_3$.

Curves 1, 2, and 3 are rates of acid formed by salivary sediment obtained from separate individuals in the absence of NaF and with 19 ppm fluoride ion. Controls 1, 2, and 3 show the corresponding rates of acid formation without added sucrose.

Fig. 5.—Inhibition of acid formation by salivary sediment with fluoride.

System: salivary sediment (2.0 ml.), 1×10^{-2} M sucrose and NaF to give the concentrations indicated in ppm. Control manometer cup contained no added sucrose or fluoride.

(b) With sediment from individual samples of saliva: In Figs. 4 and 5 are shown the results obtained with salivary sediment centrifuged out of single samples of saliva. Fig. 4 shows the effect of 19 ppm of fluoride ion with 3 samples of salivary sediment. In each case there was no inhibition of acid production. Using a range of fluoride ion concentrations (Fig. 5), it can be seen that 16 ppm had no effect whereas 32 and 64 ppm were increasingly effective. Owing to the solubility of calcium fluoride being 16 ppm, the maximum fluoride ion concentration in the presence of excess Ca^{++} should be 8 ppm.

Reversal and Enhancement of Fluoride Inhibition of Acid Formation.—1. The effect of calcium ions: The inhibitory effect of fluoride may be reversed by calcium ions. In these experiments the fluoride ion concentration was chosen to give considerable inhibition of acid production so that the effect of Ca^{++} would

be clearly shown. With 76 ppm fluoride ion (4×10^{-3} M), addition of 1×10^{-3} M Ca^{++} completely reversed inhibition by fluoride whereas 2.5×10^{-4} M Ca^{++} caused only a partial reversal.

The competitive nature of the calcium-fluoride relationship is shown in Fig. 6 where similar rates of acid formation are shown by (a) 20×10^{-4} M F^- with 5×10^{-4} M Ca^{++} and 8×10^{-3} M F^- with 2×10^{-3} M Ca^{++} and by (b) 20×10^{-4} M F^- with 2.5×10^{-4} M Ca^{++} and 8×10^{-3} M F^- with 1×10^{-3} M Ca^{++}. The ratio of F:Ca in (a) was 4:1 and in (b) 8:1. A fourfold increase in F^- concentration required a similar increase in Ca^{++} to maintain the same degree of inhibition of acid formation. The amounts of Ca^{++} used alone had no effect on the rate of acid formation from sucrose.

2. The effect of magnesium ions: Unlike calcium, magnesium ions enhance the inhibitory action of fluoride. Magnesium ion concentrations of 2×10^{-3} M and 1×10^{-3} M had no influence on the effect of 4×10^{-3} M fluoride. However, greater concentrations of Mg^{++}, such as 4×10^{-3} M and 8×10^{-3} M, did enhance the fluoride inhibition (Fig. 7). In the same experiment 1×10^{-3} M Ca^{++} completely reversed the effect of the fluoride and Mg^{++} alone did not influence the rate of acid production.

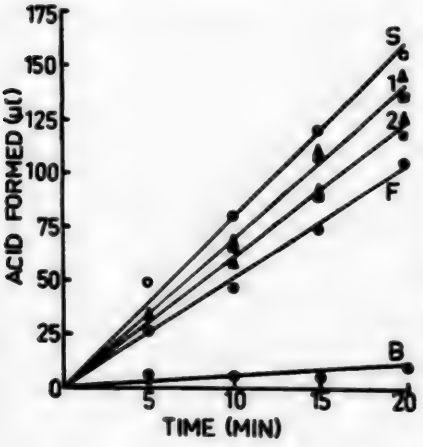

Fig. 6.

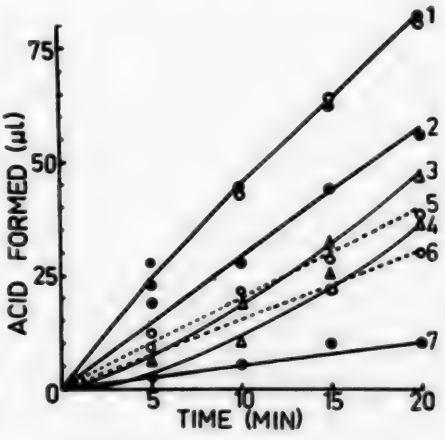

Fig. 7.

Fig. 6.—The effect of calcium ions on fluoride inhibition of acid formation by salivary sediment.

System: sediment (0.8 ml.) in 4×10^{-3} M $NaHCO_3$, 0.2 ml. 0.1 M sucrose, 0.1, 0.2 or 0.4 ml. 4×10^{-3} M NaF, 2×10^{-3} M $Ca(NO_3)_2.4H_2O$ and 4×10^{-3} M $NaHCO_3$ to 2.0 ml. Solutions of sucrose, NaF and $Ca(NO_3)_2.4H_2O$ were prepared with 4×10^{-3} M $NaHCO_3$.

Curve S, sucrose only added; Curve 1, 20×10^{-4} M F^- with 5×10^{-4} M Ca^{++} and 8×10^{-3} M F^- with 2×10^{-3} M Ca^{++}; Curve 2, 20×10^{-4} M F^- with 2.5×10^{-4} M Ca^{++} and 8×10^{-3} M F^- with 1×10^{-3} M Ca^{++}; Curve F, 2×10^{-3} M F^- (38 ppm); Curve B, no added fluoride or substrate.

Fig. 7.—The effect of magnesium and manganese ions on fluoride inhibition of acid formation by salivary sediment.

System: contained salivary sediment (approx. 5 mg. dry wt.), 1×10^{-3} M sucrose and other additions in 4×10^{-3} M $NaHCO_3$.

Curve S, sucrose alone; Curve 1, 4×10^{-3} M NaF (76 ppm F^-) + 1×10^{-3} M Ca^{++}; Curve 2, 4×10^{-3} M NaF; Curve 3, 4×10^{-3} M NaF + 8×10^{-3} M Mg^{++} as $MgSO_4.7H_2O$; Curve 4, 4×10^{-3} M NaF + 4×10^{-3} M Mg^{++} as $MgSO_4.7H_2O$; Curve 5, 4×10^{-3} M NaF + 1×10^{-3} M Mn^{++} as $MnSO_4.4H_2O$; Curve 6, 4×10^{-3} M NaF + 4×10^{-3} M Mn^{++} as $MnSO_4.4H_2O$; Curve 7, control without added substrate.

3. The effect of manganese ions: The concentrations of Mn^{++} used (1×10^{-3} M and 4×10^{-3} M) both enhanced the effect of fluoride (Fig. 7).

4. The effect of orthophosphate: Addition of 5×10^{-3} M orthophosphate to salivary sediment slightly increased the rate of acid formation from sucrose. This concentration of inorganic phosphate is similar to the values in saliva.

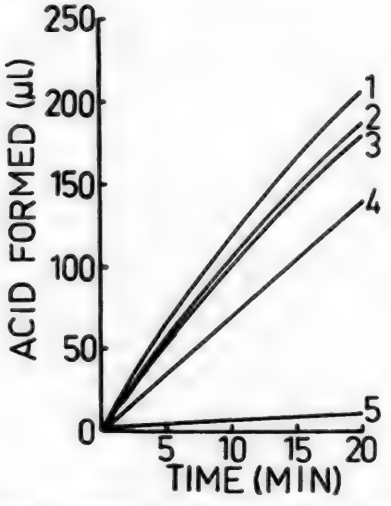

Fig. 8.—The effect of phosphate and fluoride on acid formation by salivary sediment. *System:* salivary sediment, 1×10^{-2} M sucrose and other additions in 4×10^{-3} M $NaHCO_3$.

Curve 1, 5×10^{-3} M Na_3PO_4 or 5×10^{-3} M Na_3PO_4 + 2×10^{-3} M Ca^{++} + 2×10^{-3} M NaF (38 ppm F^-); Curve 2, 5×10^{-3} M Na_3PO_4 + 0.5 ppm F^-; Curve 3, 5×10^{-3} M Na_3PO_4 + 1.0 ppm F^-; Curve 4, 5×10^{-3} M Na_3PO_4 + 38 ppm F^-; Curve 5, no added sucrose.

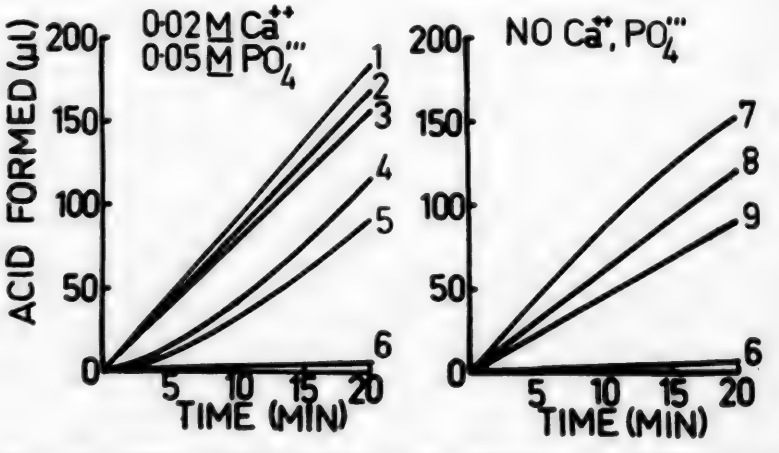

Fig. 9.—The inhibitory effect of fluoride on acid formation in the presence of Ca^{++} and $PO_4^{\equiv}$.

System: salivary sediment, 1×10^{-2} M sucrose and additions were all prepared in 4×10^{-3} M $NaHCO_3$ and volume made up to 2.0 ml. with $NaHCO_3$.

Curve 1, 0.5, 1.0 and 2.0 ppm F^-; Curve 2, nil and 3.0 ppm F^-; Curve 3, 19 and 38 ppm F^-; Curve 4, 76 ppm F^-; Curve 5, 152 ppm F^-; Curve 6, control without F^- and sucrose; Curve 7, nil, 0.5, 1.0 and 2.0 ppm F^-; Curve 8, 19 ppm F^-; Curve 9, 38 ppm F^-.

Furthermore, addition of fluoride produced slight inhibition of acid formation, even with concentrations of 0.5 and 1.0 ppm (Fig. 8). When 2×10^{-3} M Ca^{++} was present the inhibition caused by 38 ppm fluoride was completely reversed.

5. The effect of Ca^{++} and orthophosphate: Using 2×10^{-3} M Ca^{++} and 5×10^{-3} M $PO_4^{\equiv}$ the inhibitory action on acid formation was observed with a series of fluoride concentrations. A similar series of fluoride concentrations were used without addition of Ca^{++} and $PO_4^{\equiv}$. When these ions were present, 0.5, 1.0, 2.0, 3.0, 19 and 38 ppm fluoride did not significantly affect the rate of acid formation from sucrose, whereas 76 and 152 ppm F^- did inhibit the process. In the absence of additions of Ca^{++} and $PO_4^{\equiv}$ 0.5, 1.0 and 2.0 ppm F^- produced no inhibitory effect but 19 and 38 ppm F^- were inhibitory (Fig. 9).

DISCUSSION

The results obtained do not support the claims of previous investigators who brought forward evidence for the inhibitory effect of fluoride ion at concentrations of 0.5 to 1.0 ppm, i.e., 2.6×10^{-5} M and 5.2×10^{-5} M. The present studies show only slight inhibition with a fluoride ion concentration of 32 ppm (1.66×10^{-3} M) in the absence of Ca^{++} and $PO_4^{\equiv}$ concentrations similar to those found in saliva. However, in the presence of these amounts of Ca^{++} and $PO_4^{\equiv}$ 38 ppm F^- did not inhibit acid formation.

In the report of Wright and Jenkins,[1] the differences in pH recorded for incubated saliva-glucose mixtures are very small and could be within experimental error. Furthermore it is not possible to evaluate the differences in titration figures given, as the actual values from which these figures were determined were not shown. The other claim for inhibition by fluoride on acid formation by oral bacteria (Bibby and Van Kesteren[2]) may also be criticized on the following points. First, it is only after growth has ceased that fluoride inhibits acid formation in pure cultures of lactobacilli and streptococci. During logarithmic growth, 45 ppm F^- had no effect on acid formation. Second, the figures given for acid formed in a given time are averages of numerous tests with a number of different strains of each species of bacterium. The range of acid formed with up to 100 ppm NaF in 24 hours with lactobacilli and in 8 hours with streptococci was just as great as when no fluoride was added.

Fluoride ions inhibit a number of enzyme systems (Borei[7]). In the glycolytic mechanism the most sensitive enzyme to fluoride is enolase which catalyses the conversion of 2-phosphoglyceric acid to phospho-enolpyruvic acid. This enzyme requires magnesium ions for its activity. In the presence of fluoride and orthophosphate, magnesium fluorophosphate is formed which competes with magnesium ions for the enzyme surface. Magnesium fluorophosphoenolase is formed which is inactive. Magnesium ions enhance the inhibitory action of fluoride on enolase as also does Mn^{++} and Zn^{++} (Warburg and Christian[8]). The effects of Ca^{++}, $PO_4^{\equiv}$, Mg^{++} and Mn^{++} on fluoride inhibition of acid formation by salivary sediment is in agreement with the known effects of these divalent metallic ions and of phosphate on enolase. In saliva the concentration of Ca^{++} and orthophosphate is sufficient to prevent inhibition of acid formation by concentrations of up to 38 ppm F^-.

SUMMARY

The anti-enzymatic action of fluoride on oral bacteria as salivary sediment has been studied. Fluoride ion concentrations of 0.5 and 1.0 ppm did not inhibit the rate of acid formation from glucose and sucrose. At values of 32 ppm fluoride, inhibition of acid formation was slight (10 to 30 per cent) in the absence of added Ca^{++} and $PO_4^{\equiv}$. Addition of Ca^{++} in sufficient concentration completely reversed the inhibition of fluoride, whereas Mg^{++} enhanced the inhibitory action as did Mn^{++} and $PO_4^{\equiv}$. These observations are in agreement with the known effects of these divalent ions and of orthophosphate on the inhibition of enolase by fluoride. In the presence of 2×10^{-3} M Ca^{++} and 5×10^{-3} M $PO_4^{\equiv}$, 0.5, 1.0, 2.0, 3.0, 19, and 38 ppm F^- had no effect on the rate of acid formation from sucrose.

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DETERMINATION OF FLUORIDE IN ORGANIC AND INORGANIC FRACTIONS OF DENTIN AND ENAMEL

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REPORTS in the literature suggest that certain elements existing within the tooth are distributed between the organic and inorganic phases. Koss and Ginn[1] reported that some 87 per cent of the total sodium content is extracted with the organic portion of the tooth substance by means of alkaline ethylene glycol solution; and Rosebury[2] has shown that iron is associated with a protein in enamel.

Sufficient evidence has been presented by Fischer and Muhler,[3] Gerould,[4] and Scott, Picard, and Wyckoff[5] to justify the conclusion that either calcium fluoride, calcium fluoroapatite, or a combination of these, is formed when enamel is treated with certain fluoride compounds, notably sodium fluoride. However, to the knowledge of the present authors no work has been done to show whether or not the interaction of fluoride ion and tooth substance is limited to the production of calcium fluoride and calcium fluoroapatite. The present investigation was undertaken in order to demonstrate in which fractions of the tooth fluoride could be found.

EXPERIMENTAL

Materials.—Teeth used in these experiments were procured from dentists* practicing in Green Bay, Wisconsin, a city which has a water supply with a natural content of 2.1 ppm. fluoride. It was requested that the dentists collect teeth only from lifetime residents of Green Bay and not preserve the teeth chemically, but refrigerate them until a sufficient supply was obtained for mailing. Only teeth free from macroscopic or clinical caries were used in the experiments.

Sample Preparation.†—Experimentation was done on (1) whole teeth, (2) whole crowns, (3) crown dentin, (4) root dentin, and (5) enamel. In each case the tooth was first scrubbed clean with a nylon-bristled denture brush, using floured pumice as the cleaning agent.

Whole teeth were crushed in a steel, plunger type mortar and pestle to the selected mesh size.

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†Technical assistance was rendered by Isadore Ettleman, DT3, USN, in the preparation of tooth samples.

Whole crowns were severed at the cementoenamel junction before being pulverized in the mortar.

Crown dentin was prepared by first embedding the root end of the tooth (to the cementoenamel junction) in dental stone. The exposed enamel was then removed by using a diamond disk, and the dentin cut off at the cementoenamel junction. This dentin was then crushed as described above.

Root dentin was prepared by severing the tooth at the cementoenamel junction and then cleaning debris from the pulp chamber and root canals before crushing the dentin in the mortar. No attempt was made to remove the cementum.

Enamel samples were prepared by embedding the crown (to the cementoenamel junction) in dental stone. The exposed root was then removed with a diamond disk and discarded. The crown dentin was removed by using dental burrs of sizes 8, 2, and ½. The enamel was then removed from the dental stone, recleaned, and crushed in the mortar.

All cutting and burring operations were done with slow speed in order to minimize excessive heat production.

Procedures.—Decalcification was carried out in an apparatus which has been described by Losee, Leopold, and Hess.[6] The apparatus consisted of a shielded adapter over the large mouth of which two sheets of nonwaterproof cellophane were secured by three separate ties. The sample to be decalcified was then introduced into the small mouth of the adapter and the sample washed onto the inner surface of the dialyzing membrane with a small quantity of the demineralizing solution. The adapter was next placed in a 600 ml. beaker containing the main volume of solution used for demineralization. The adapter was adjusted so that the inner and outer fluid levels were the same. Fresh demineralizing fluid was used to replace the old at the end of a 24-hour period, and this in turn was replaced with distilled water after another 24 hours. Continuous stirring of the outside solution was done throughout the decalcification and rinsing processes. The contents of the dialysis chamber were removed after a second 24-hour period of rinsing, and both residue and internal fluid were analyzed for presence of any residual calcium (oxalate method). After ascertaining that decalcification was complete the inner contents were analyzed for fluoride. In some cases separate determinations were done on supernatant and residue, but usually one determination was done on the total combined phases (entire contents of demineralization chamber).

Ethylene Glycol-KOH Extraction. Extraction of the organic fraction was done on dentin, enamel, and whole tooth samples according to the technique of Crowell, Hodge, and Line.[7] At the end of the extraction period the inorganic residue was separated from the glycol supernatant by centrifugation and the separated phases were analyzed for presence of fluoride.

Acetone-Alcohol Extraction. A sample of dentin was extracted at room temperature to remove the lipid fraction. This extraction was made using a solution consisting of equal volumes of acetone and 95 per cent ethanol. After

2 days the mixture was filtered through sintered glass and the filtrate evaporated. A fluoride determination was made on the residue from the evaporated solvents.

Analytical methods used in the determination of fluoride were those of Gettler and Ellerbrook,[8] Ieken and Blank,[9] and Megregian.[10] Two of these methods[9, 10] became available during the course of this study. Cross checks between all 3 methods gave results which agreed to within 1 per cent. Megregian's method was the one finally adopted and used for the majority of the determinations.

Ashing of enamel and whole tooth samples was done in order to compare the fluoride content of the residue with that of the original unashed sample. The electric muffle temperature used was 550° C. for three hours' duration.

RESULTS

Fluoride Content of Protein Residues After Demineralization.—(Table I) Two samples of dentin, one containing 640 μg fluoride per gram and the other containing 940 μg fluoride per gram initially, were decalcified with an aqueous solution of 5 per cent ethylenediamine tetracetic acid,* adjusted to a pH of 7.2. A portion of the sample containing 640 μg fluoride per gram initially was also decalcified with a solution of 5 per cent trichloroacetic acid. Protein residues thus obtained were found to be devoid of fluoride.

TABLE I
DECALCIFICATION

TYPE OF SAMPLE	MESH SIZE	CONCENTRATION OF FLUORIDE IN SAMPLE INITIALLY (μg/GM.)	WT. SAMPLE USED FOR DECALCIFICATION (GM.)	DECALCIFYING AGENT	VOLUME AGENT USED (ML.)	pH		FLUORIDE FOUND IN PROTEIN RESIDUE (μg)
						INITIAL	FINAL	
Crown dentin	160	640	1.04	5% EDTA	1000	7.2	7.2	0
Crown dentin	160	640	0.904	5% Trichloroacetic acid	500	--	--	0
Root dentin	200	940	1.69	5% EDTA	1200	7.2	7.2	0
Enamel	200	90	1.0	0.1 N HCl	1000	1	1	0

One sample of enamel, containing 90 μg fluoride per gram initially, also was decalcified using 0.1 N HCl. Sufficient volume of the HCl was used to obtain a final pH of 1. The organic residue contained no fluoride.

Fluoride Content of Separate Phases After Extraction With 3 Per Cent Potassium Hydroxide in Ethylene Glycol.—(Table II) The following samples were used for extraction with alkaline ethylene glycol:

1. Two samples of dentin, one containing 940 μg fluoride per gram and the other containing 440 μg fluoride per gram initially.

2. One sample of whole crown containing 175 μg fluoride per gram initially.

*Supplied through the courtesy of Bersworth Chemical Co., Framingham, Mass., and Alrose Chemical Co., Providence, R. I.

TABLE II
EXTRACTION WITH 3% KOH IN ETHYLENE GLYCOL

TYPE OF SAMPLE	MESH SIZE	CONCENTRATION OF FLUORIDE IN SAMPLE INITIALLY (μg/GM.)	WT. SAMPLE USED FOR EXTRACTION (GM.)	VOLUME KOH-GLYCOL SOLUTION (ML.)	FLUORIDE CONTENT OF SEPARATE PHASES (PER GM. EXTRACTED)	
					RESIDUE (μg)	EXTRACT (μg)
Root dentin	200	940	0.47	30	930	0
Crown dentin	160	440	0.77	30	440	0
Whole crown	100	175	2.0	30	182	0
Whole teeth	200	515	2.0	30	465*	46*
Whole teeth	200	440	1.0	30	443	0

*Average of two values.

3. Two samples of whole teeth, one consisting of both molars and incisors, contained 515 μg fluoride per gram initially, and the other, consisting of incisors only, contained 440 μg fluoride per gram initially.

The fluoride content of all the residue, with the exception of that whole tooth sample containing 515 μg fluoride per gram initially, was in good agreement with the concentrations of fluoride in the samples initially.

The extracts were devoid of fluoride, with the exception of that extract from the whole tooth sample containing 515 μg fluoride per gram initially. In this instance, 46 μg fluoride per gram sample extracted was found in the extract, and 465 μg fluoride was found in the residue; the sum of these values was 511 μg fluoride per gram sample extracted.

The finding of fluoride in this one extract, not being consistent with the absence of fluoride found in extracts of other whole tooth samples, suggested the need for repetition of this phase of the study. Another portion of the same sample was extracted with alkaline ethylene glycol and, on determination of the fluoride content of the extract, a value of the same magnitude was obtained. The deviation of these results from those obtained on extracts of the other 4 tooth samples is unexplained at the present time.

Fluoride Content of Acetone-Alcohol Extract.—The sample of dentin used for this experiment contained 940 μg fluoride per gram initially.

The extract contained no fluoride.

Fluoride Content of Ashed Samples.—A sample of enamel, containing 94 μg fluoride per gram initially, was ashed, as was also a sample of whole teeth, containing 440 μg fluoride per gram initially.

The fluoride contents of the ashed samples were found to represent the total fluoride in the original samples used for ashing.

SUMMARY AND CONCLUSIONS

Tooth samples were obtained from an endemic fluoride area and the fluoride content determined before experimentation. Various means were used to separate the organic and inorganic fractions of these samples. This was followed by a quantitative determination of fluoride in the separated phases.

The results strongly indicate that most, if not all, fluoride which is to be found in human teeth is associated with the inorganic fraction.

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STUDIES ON THE PHYSICOCHEMICAL PHENOMENA RELATED TO DENTAL CARIES

III. The Effect of Sucrose Upon the Solubility of Human Dental Enamel

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SOLUBILITY studies on human dental enamel have engaged the attention of many investigators.[1-75] While the methods used have varied widely the purpose of most of the studies was to learn about the caries process or how to prevent it. Many investigations have dealt with the dissolving effect of various solutions; most of the solutions used were acidic in nature. The belief that carbohydrates and, more specifically, products of carbohydrate degradation were involved in caries formation gradually gained prominence over a span of years. This idea stimulated further research involving the effect of carbohydrate fermentation upon enamel. In addition, many investigators have sought to treat enamel to render it less soluble in acidic solutions. The results of these efforts by many workers have been to establish that carbohydrates serve as the main source for acid formation by fermentation and that this acid is directly involved in the decalcification process. It also has been shown that many substances (the majority of which are fluoride compounds) when applied to the enamel surface diminish the acid solubility of that surface. In none of the many studies, however, has the possible effect of sucrose per se on the enamel solubility been emphasized.

The purpose of the present paper is to report a study which was designed to test the effect of sucrose on tooth enamel solubility in acid solutions.

Sugars are accepted by most investigators as the principal substrate for acid formation in the mouth and thus are seen to play a direct role in dental caries. It is possible that sugars may affect the dental caries process in other ways in addition to that of acting as a source of acid. Such a possibility has prompted or influenced several investigations. For example, the effect of sucrose and other sugars upon tooth permeability has been studied.[76-78] The effect of sucrose upon ion diffusion through semipermeable membranes also has been investigated.[79-81] The latter studies showed that sucrose changes the diffusion rates of hydrogen ions, calcium ions, and phosphate ions. This may possibly indicate that sucrose influences factors affecting the development of dental caries and therefore it is of interest to determine further influences of a physicochemical nature exerted by sucrose on the process of caries development. Preliminary studies in this laboratory[82] showed a definite

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influence of sucrose upon enamel solubility. The present work indicates that sucrose has a retarding effect on the rate of enamel dissolution by lactate buffer solutions.

EXPERIMENTAL METHODS AND MATERIALS

The effect of sucrose upon the solubility of powdered human dental enamel was tested by shaking samples of the powdered enamel in each of the several solutions for varied time intervals following which the solutions were tested for calcium (and, in some cases, for phosphate content). The amounts of calcium appearing in solution were taken as an index of the rate of enamel dissolution in the several solutions. In addition, phosphate determinations were made on the same solutions at 3 of the pH levels.

The dental enamel used in the present work was the homogeneous mixture of a pooled sample of ground enamel with a particle size ranging from 270 to 150 mesh. The enamel was obtained from noncarious, clean, adult human molars which were dried for 24 hours at 100° C. The teeth were cracked in a mortar and pestle. Those pieces of crown enamel apparently free of dentin were separated by inspection, ground in a mortar and pestle and sieved. The particles smaller than 150 mesh (the holes in 150 mesh screen are approximately 0.0041 inch diameter) and larger than 270 mesh (approximately 0.0021 inch diameter) were collected and washed with a 50-50 (volume) mixture of ethyl alcohol-ethyl ether and dried. The material was subjected to the Brekhus and Armstrong[83] technique for separation of dentin and enamel in order to remove any dentin particles. All samples for the individual solubility tests were taken from one pooled sample of powdered enamel treated in the manner just described (with the exception of the samples tested in the pH 5.5 solutions which were run with a separate sample of enamel prepared in identical manner).

The solutions used in the study were lactate buffer, lactate buffer with 0.5 M sucrose, and lactate buffer with 1.0 M sucrose. In all cases the total lactate present was 1 mole per liter. Sufficient NaOH was added to the 1 M lactic acid in the preparation of each solution to obtain the desired pH. All solutions were preserved by addition of a small amount of mercuric iodide and by storage in the refrigerator until used. The above 3 types of solutions were prepared at pH values of 4.0, 4.5, 5.0, 5.5, and 6.0.

The solubility tests were run by shaking individual 50 mg. samples of the powdered dental enamel with 20 ml. of the desired solution in screw-cap vials at 37° C. The vials were shaken in a thermostated water bath with constant agitation for varied time intervals of 5, 10, 15, 30, 60, 120, and 240 minutes. Separate vials of solution were used for each time interval and in most cases duplicate vials were used at each time interval. Duplicate analyses were made on the contents of each vial. All vials in a given run were started at the same time and at the appointed intervals vials were removed from the incubating bath and the contents immediately filtered with suction to separate the remaining undissolved enamel from the solution in order to stop the process of dissolution. Filtration was accomplished by suction with Buchner filter funnels.

Samples of the filtrate were taken for calcium and phosphorus analyses. The calcium determinations were made on all samples by the Clark-Collip[84] modification of the Kramer-Tisdall method which involves the precipitation of calcium as the oxalate followed by the titration of the oxalate with standardized potassium permanganate solution. The phosphorus determinations, which were made on the pH 5.0, 5.5, and 6.0 buffer solutions, were carried out with a modification of the colorimetric method of Fiske and Subbarow.[85]

In a separate experiment, soluble calcium ions in the presence of 0.5 M and 1.0 M sucrose were determined with the above-mentioned method in order to ascertain that the observed differences in calcium content in the enamel solutions were real and not artifacts. That is, it was necessary to rule out the possibility that sucrose was affecting the rate or degree of precipitation of calcium oxalate. For this purpose calcium chloride solutions were prepared with calcium concentrations of 10.0 and 50.0 mg. per 100 ml. of solution. Such solutions were made with no sucrose as well as with 0.5 M and 1.0 M sucrose. Upon addition of ammonium oxalate to the various solutions it was apparent that the rate of precipitation of calcium oxalate was slightly slower in the presence of 0.5 M sucrose and slower in the presence of 1.0 M sucrose. However, the precipitation was complete in all cases in less than 1 hour. Samples standing for 1 hour and those standing overnight showed the same results. The viscosity and density of the 1.0 M sucrose solutions were noticeably greater than in the other solutions so that mixing of the ammonium oxalate solution and the sucrose solution was slower. This could have resulted in low calcium results if the 2 solutions were not mixed and if they were not allowed to stand for precipitation for at least 1 hour. All of the solutions in the experimental runs with dental enamel were mixed with the ammonium oxalate solution when the latter was added and all of the solutions were allowed to precipitate well over 1 hour (many of them stood overnight before the supernatant fluid was removed following centrifugation). Thus it can be stated definitely that the differences in calcium content of the solutions following incubation with enamel were real differences and not due to incomplete precipitation of calcium oxalate in the presence of sucrose. This is further supported by the fact that the amount of phosphate ion going into solution paralleled the calcium.

Finally, the effect of sucrose alone upon enamel solubility was tested. Human dental enamel samples prepared as described above but of smaller particle size (270 mesh and finer) were shaken for 24 hours with distilled water and with 1 M sucrose. One gram samples of the enamel were shaken at room temperature (27 to 29° C.) with 100 ml. of distilled water and with 100 ml. of 1 M sucrose. Both solutions were adjusted to pH 7.0 (by addition of minute amounts of NaOH) before the enamel was added. After 24 hours the pH of both solutions remained essentially unchanged (water, pH 6.92; sucrose, 6.95). Both solutions were centrifuged (20,000 rpm, 20 min.) to remove

suspended particles of enamel and aliquots of solution taken for calcium determination as before. Little enamel was dissolved by either solution; however, the amount dissolved by the sugar solution was slightly higher.

RESULTS

The numerical results are presented in the tables. Fig. 1 shows graphically the effect of time on the solubility of human dental enamel in the various buffer solutions (the content of calcium ion in solution at the given time being taken as the index of solubility). In the figure the sets of 3 times for each pH level are joined by hatch lines. (The 4-hour figures given in Table I were not plotted in the graph in order to show the first 2-hour results in more detail.)

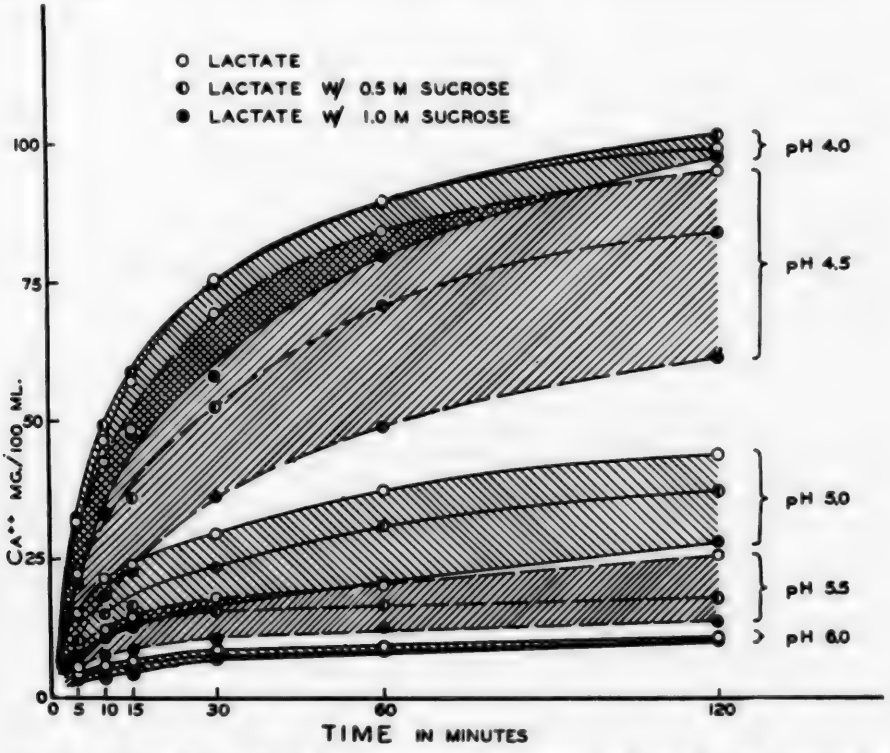

Fig. 1.—Rate of dissolution of powdered dental enamel in lactate buffer solutions as measured by Ca++ content of the solutions. The three solutions at each pH level are connected with cross-hatched lines.

The curves all show a deceleration of the dissolution rate with time as noted in other studies.[30, 66] This is due to the retarding effect of the increasing amounts of calcium and phosphate ions in solution with time as well as to the rapid dissolution of the smaller particles with the resulting decrease in total enamel surface. The effect of the sugar on the rate of enamel dissolution is less marked in the pH 4 and pH 6 solutions than in the rest. In all of the

pH 4 solutions the rate of dissolution is quite high while at pH 6 the rate is low. In the pH 4 solutions most of the enamel was dissolved by the end of 1 hour. In almost all cases it can be seen that the presence of sugar in the buffer solution reduced the amount of calcium (and phosphate) going into solution. From the standpoint of comparison of the present results with possible in vivo conditions of enamel decalcification the most important solutions are those of pH 4.5, 5.0, and 5.5 since a pH of 4.0 on the tooth surface would not be expected to occur very frequently and at a pH of 6.0 the rate of decalcification is expected to be almost negligible. The magnitude of the sugar effect on the dissolution rate can be assessed by reference to Table I. For example, at pH 4.5, the amount of calcium in solution after 5 minutes in the lactate buffer (32.0 mg./100 ml.) is approximately equal to that found after 10 minutes in the lactate buffer containing 0.5 M sucrose (33.4) and after 30 minutes the amount dissolved in the presence of 1.0 M sucrose is only slightly more (36.4). Likewise the amounts dissolved in lactate buffer, 0.5 sucrose +

TABLE I
ENAMEL DISSOLVING EFFECT OF BUFFER SOLUTIONS WITH AND WITHOUT SUCROSE AS MEASURED BY DISSOLVED CALCIUM (MG. CA/100 ML. SOLUTION)

pH	SOLUTION	TIME IN MINUTES 5	10	15	30	60	120	240
	Lactate	31.3	46.3	57.2	75.6	89.8	99.6	111
4.0	0.5 M Sucrose in Lactate	32.6	49.5	58.9	74.9	89.9	102	111
	1.0 M Sucrose in Lactate	20.5	33.2	47.8	58.2	79.9	98.0	115
	Lactate	32.0	42.8	48.6	69.7	84.5	95.4	108
4.5	0.5 M Sucrose in Lactate	22.3	33.4	36.2	52.7	71.2	84.4	94.6
	1.0 M Sucrose in Lactate	15.0	18.3	22.7	36.4	49.1	61.6	80.5
	Lactate	15.2	21.4	23.8	29.7	37.9	44.4	50.8
5.0	0.5 M Sucrose in Lactate	10.0	15.0	16.6	23.5	31.2	38.0	42.5
	1.0 M Sucrose in Lactate	8.2	10.7	12.6	16.0	20.9	28.5	33.8
	Lactate	9.6	12.2	14.7	18.0	20.2	26.0	31.2
5.5	0.5 M Sucrose in Lactate	9.8	12.1	14.4	15.4	16.8	18.0	18.8
	1.0 M Sucrose in Lactate	5.3	7.5	8.6	10.6	12.8	13.9	16.9
	Lactate	5.6	5.8	6.6	8.9	9.4	11.4	12.3
6.0	0.5 M Sucrose in Lactate	4.6	5.0	5.2	7.1	8.6	10.6	11.6
	1.0 M Sucrose in Lactate	3.4	3.9	4.1	7.4	8.4	10.9	12.4

TABLE II
ENAMEL DISSOLVING EFFECT OF BUFFER SOLUTIONS WITH AND WITHOUT SUCROSE AS MEASURED BY DISSOLVED PHOSPHORUS (MG. P/100 ML. SOLUTION)

pH	SOLUTION	TIME IN MINUTES 5	10	15	30	60	120	240
	Lactate	6.0	9.0	9.7	12.8	16.1	19.0	21.4
5.0	0.5 M Sucrose in Lactate	3.3	5.3	6.6	9.8	12.6	15.3	17.6
	1.0 M Sucrose in Lactate	2.3	2.9	3.1	4.7	7.6	10.2	12.3
	Lactate	3.9	5.1	6.0	7.2	9.2	10.4	11.7
5.5	0.5 M Sucrose in Lactate	3.6	4.7	5.2	5.9	6.7	7.2	7.5
	1.0 M Sucrose in Lactate	2.1	2.6	4.1	4.8	5.7	6.4	7.2
	Lactate	1.5	1.6	2.2	2.4	3.3	3.7	4.1
6.0	0.5 M Sucrose in Lactate	1.6	2.0	2.0	2.8	2.9	3.7	4.4
	1.0 M Sucrose in Lactate	1.2	1.5	2.0	2.4	3.0	4.0	4.6

lactate buffer, and 1.0 M sucrose + lactate buffer are about equal at 15 minutes (48.6), 30 minutes (52.7) and 60 minutes (49.1), respectively. These examples are sufficient to show the effect of the presence of sucrose on the dissolving action of the buffer solutions. The results on phosphate determinations parallel reasonably well those of the calcium; however, there was little difference found between the 3 solutions at pH 6.0.

The calcium/phosphorus weight ratios were found to vary considerably but in no particular pattern. The extreme values were 2.0 and 4.1, the mean value was 2.70 with a standard deviation of 0.4. These values are above the Ca/P weight ratio consistently found in this laboratory for pooled human dental enamel which approximate 2.0 (e.g., Suess,[66] 2.01; and Young,[82] 1.96). The generally accepted literature values approximate 2.0 also as shown by the work of Armstrong and Brekhus[86] who found the ratio on carefully purified enamel to be 2.03.

TABLE III
Ca/P WEIGHT RATIO

pH	SOLUTION	TIME IN MINUTES 5	10	15	30	60	120	240
	Lactate	2.5	2.4	2.5	2.3	2.4	2.3	2.4
5.0	0.5 M Sucrose in Lactate	3.0	2.8	2.4	2.4	2.5	2.5	2.4
	1.0 M Sucrose in Lactate	3.6	3.7	4.1	3.4	2.8	2.8	2.7
	Lactate	2.5	2.4	2.4	2.5	2.2	2.5	2.7
5.5	0.5 M Sucrose in Lactate	2.7	2.6	2.8	2.6	2.4	2.5	2.5
	1.0 M Sucrose in Lactate	2.5	2.9	2.1	2.2	2.3	2.2	2.2
	Lactate	3.7	3.6	3.0	3.7	2.8	3.1	3.0
6.0	0.5 M Sucrose in Lactate	2.9	2.5	2.6	2.5	3.0	2.9	2.6
	1.0 M Sucrose in Lactate	2.8	2.6	2.0	3.1	2.8	2.7	2.7

Sucrose alone, in neutral aqueous solution, tends to increase enamel solubility as shown by the results obtained when enamel was shaken with water and with 1 M sucrose. The amounts of calcium in solution at the end of 24-hour exposure of the finely powdered enamel (<270 mesh) to the solutions were 1.67 ± 0.20 mg. Ca^{++}/100 ml. of 1 M sucrose and 1.19 ± 0.15 mg. Ca^{++}/100 ml. of distilled water. The figures ±0.20 and ±0.15 represent standard deviations from the mean of 4 determinations each and reflect the variation encountered with low amounts of calcium. The probability of the 2 average figures, 1.67 and 1.19, occurring by chance alone is roughly 1 in 20.

DISCUSSION

Several possible explanations exist for the observed effect of sucrose in lowering the dissolution rate of enamel particles in buffered acid solutions. These can be classified as: (1) viscosity effects, (2) coating effect, (3) chelation or complexing effect, and (4) hydration effects.

The viscosities of the buffer solutions containing 0.5 M sucrose and 1.0 M sucrose are considerably greater than the viscosities of the solutions with no sugar. The increased viscosity would be expected to diminish the rates of diffusion of the various ions present. As shown by Strålfors,[65] increased vis-

cosity in the surrounding solution results in a lower rate of dissolution of enamel due to the increase in amount of saturated solution surrounding the enamel surface (i.e., saturated with respect to calcium and phosphate ions). Both the rate of diffusion of calcium and phosphate ions away from the enamel surface and the diffusion rate of hydrated protons toward the surface are diminished in the more viscous sugar solutions. This effect should operate in an identical fashion with intact or with powdered enamel of equal total surface area. It is of interest in this connection to recall that Forscher[79-81] found that the rate of ion diffusion through a semipermeable membrane was diminished in the presence of sucrose. In another respect, however, the results obtained with powdered enamel are not directly comparable with in vivo conditions. The dissolution rate of the enamel particles depends, as discussed above, upon the rate of diffusion of ions to and from the particle surface; this rate will be affected by relative movement of the enamel particles and the surrounding solution. The increased viscosity in the presence of sucrose could materially reduce this rate of mixing. The reduction in solubility due to this effect, the magnitude of which is unknown, would not be expected to operate in vivo.

Another mechanical or physical action by which sucrose would be expected to reduce the rate of enamel dissolution is the coating or layering of the enamel particles by the sugar, or hydrated sugar, molecules. The magnitude of this effect is difficult to assess but it might be expected to be reasonably small since the sugar molecule in acid solutions would undergo only an infinitesimal amount of ionization and then would have practically no ionic charge. The sugar molecules through their many –OH groups could be attracted by van der Waal's forces to the ions in the enamel surface either directly or through water molecules. That such a phenomenon does exist seems probable in light of the effect shown by sucrose (vide supra) in slowing the rate of precipitation of calcium oxalate. In vivo the coating effect of sucrose might be expected to be less due to the presence on the tooth surface of many molecular and ionic species which would leave less room to be occupied by sucrose molecules.

Sucrose forms compounds with calcium ions in basic solution.[87] The exact nature of the compounds is unknown; they appear to vary with the relative amounts of calcium ions and sucrose present.[87] These compounds, which are soluble, must be complexes and perhaps they are chelates. Calcium oxide and calcium hydroxide are dissolved by sucrose solutions; use has been made of this in analytical procedures[88-90] in which these materials are extracted away from calcium carbonate (which is not soluble in the sucrose solution) or from magnesium compounds. The extent of compound formation between calcium ions and sucrose may depend upon the ionization of sucrose as an acid; if this be true it appears likely that such compound formation would be extremely limited in acid solution due to the suppression of sucrose ionization. It was noted previously that at neutrality a sucrose solution, of 1 M concentration, dissolved powdered enamel slightly more than did dis-

tilled water. This is due, most likely, to the slight tendency of the sucrose to form calcium complexes at pH 7. Calcium ions chelate with a number of organic compounds[91] including lactic acid[92-94]; sucrose may exert an effect on the calcium lactate chelate by combination as suggested for the interaction of cupric chelates with sugars.[95] Such a combination, depending upon its nature, could decrease or increase the rate of enamel dissolution.

Sucrose molecules should, by binding water molecules through hydrogen-bonding, reduce the number of free water molecules and increase thereby the effective concentration of hydrogen ion in a given solution.[80] This effect is not significant in the present experiment since the buffer solutions were adjusted to the proper pH after sucrose was added. The effect is of significance in vivo but its magnitude is unknown.

The significance of the relatively high Ca/P ratio in the buffer solutions is unknown. Swartz and Phillips[67] found variable Ca/P ratios in solutions resulting from the acid dissolution of enamel from restricted areas on intact tooth surfaces. On the same area from 20 teeth immersed in acetic acid the decalcification produced solutions with Ca/P ratios ranging from 1.99 to 2.82 with an average of 2.38. Kanthak[30] found the Ca/P ratios in solution to parallel closely the ratio found in the enamel.

From the discussion of the present work it would appear that sucrose exerts a retarding effect upon the rate of enamel dissolution in lactic acid solutions which may indicate a possible influence on the rate of caries formation. Experiments on whole enamel are indicated as the next logical step in approaching more nearly in vivo conditions.

SUMMARY

The presence of sucrose in lactate buffer solutions was shown to decrease the solubility of powdered human dental enamel. It is suggested that the picture of caries development may be influenced by the presence of sucrose in plaque material acting in the dual role of (1) the main substrate for degradation to acid to cause decalcification, and (2) a retarding influence on the rate of enamel dissolution.

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RAPID ACID FORMATION RESULTING FROM THE ACTION OF PLAQUE OR SALIVARY SEDIMENT ON SUGARS

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IT HAS BEEN known that monosaccharides, disaccharides, and polysaccharides can be broken down to form acid under satisfactory conditions of temperature and aeration by bacterial or enzymatic elements present in the average human mouth.[1-4] These findings have contributed much to our newer concepts of dental caries and the origin of the carious lesion.

This study revealed that acid can be formed by the action of plaque or salivary solids on sugar in seconds. Stephan[2] had estimated that from 3 to 30 minutes were needed for active plaque to produce demineralizing concentrations of acid from sugar under ideal conditions. Beyond this, a review of the literature discloses nothing that would establish the rapidity with which demineralizing concentrations of acid can be formed to initiate the carious lesion in the presence of a normal salivary flow.

A pilot study compared the acid-producing powers of the sediment and supernatant fractions of saliva and demonstrated that much of the enzymatic acid-producing power of the saliva appeared to concentrate in the sediment fraction. Subsequent investigation has therefore centered on the acidogenic properties of salivary sediment and plaque and is the subject of this report.

ACID PRODUCTION FROM PLAQUE AND SUGAR

Forty young adult males (dental students) were employed as subjects in this study.* Despite above-average interest in dental hygiene, the range of caries-experience extended from 0 to 28 in the number of decayed, missing, or filled (DMF) teeth. The subjects worked in pairs, each examining his partner to establish his caries-experience rating (DMF) and to obtain the specimen of plaque used in this study. Plaque, as used in this study, refers to the whitish, tenaceous film remaining on the tooth surface after rinsing and which must be mechanically removed by scraping the tooth surface with a scaler or similar instrument. The following series of steps was carried out in every instance: (1) Each mouth was rinsed with distilled water for from 30 to 45 seconds to remove dislodgeable debris, residual saliva, and interfering substances which might serve to retard or accelerate the test response. (2) Plaque was scraped from tooth surfaces with a metal explorer and deposited in 2 small "mounds"

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*Ten of an original group of 50 subjects were eliminated from the study because it was felt that they had not cooperated fully in the rinsing procedure described below.

about 3 inches apart on a white, glazed tile. (3) Each "mound" was then depressed in the center with a blunt probe to form a "well" or circle about one-half inch in outside diameter. (4) The 2 plaque "wells" were then overflowed with one or more drops of a 0.1 per cent solution of aqueous methyl red.* (Any signs of orange or red coloration at this point was interpreted as indicating the presence of acid before sugar had been added, and was considered disqualifying for this study.) (5) Several tiny crystals of glucose (monosaccharide) or sucrose (disaccharide) were then dropped into 1 central "well"; and the time noted to the exact second. The second "well" remained unsugared, and served as a "control" as well as convenient basis for color comparisons. (6) Both "wells" were watched intently for the development of minute specks of orange or reddish color indicative of the minute formations of more concentrated acid. Exact clock-times were recorded along with the colors for use in estimating the time required for the development of these tiny acid spots. (Development of colored specks in the unsugared control disqualified the data obtained since it is obvious in these instances that acids or carbohydrates were already present in the specimen before the sugar was added.) (7) Time intervals in seconds elapsing from the moment sugar was added until the respective color spots were noted were then calculated, and plotted graphically against the corresponding pH values.[5]

RESULTS

No acid formation was indicated in any of the unsugared controls during the tests. Sample results obtained on a representative number of the group are presented graphically (Fig. 1). These were "selected" to demonstrate the range covered by the group. Hydrogen-ion concentration readings were also made on the salivas of the subjects to establish the pH of the environments from which the plaque specimens were taken. These determinations were made with a Beckman pH meter since the indicator solution used in the experiment shows no color change above pH 6.3. Environmental pH ranged from 6.65 to 7.8.

The range of acid-production rates varied from 15 to 210 seconds in times required to reach demineralizing acid concentrations of from pH 4.3 to pH 4.5 from an average environmental pH of about 6.8.

The first colored specks to be noticed are usually orange in color, and are distributed around the outer border of the plaque "mound." These intensify into a deep red as other orange or reddish spots appear around the margin. This continues with increasing coalescence until a definite red ring forms and spreads (Fig. 2).

*Aqueous methyl red in triple-distilled water makes an indicator solution with colors characteristic of the following hydrogen-ion concentrations:

pH 6.3 (and higher)	pale yellow
pH 5.8	buff
pH 5.3	orange
pH 4.8	orange-red
pH 4.3	red.

Manufactured by Hartman-Leddon Company, Philadelphia, Pa.

DISCUSSION

It will be evident from the representative data presented in Fig. 1 that acid concentrations equivalent to pH 4.3 or less can be rapidly formed in minute quantities by the action of plaque on sucrose or glucose. Since Stephan[2] has shown that tooth enamel can be demineralized by acid concentrations of pH 5 and under, it becomes evident that plaque and sugar can produce acids capable

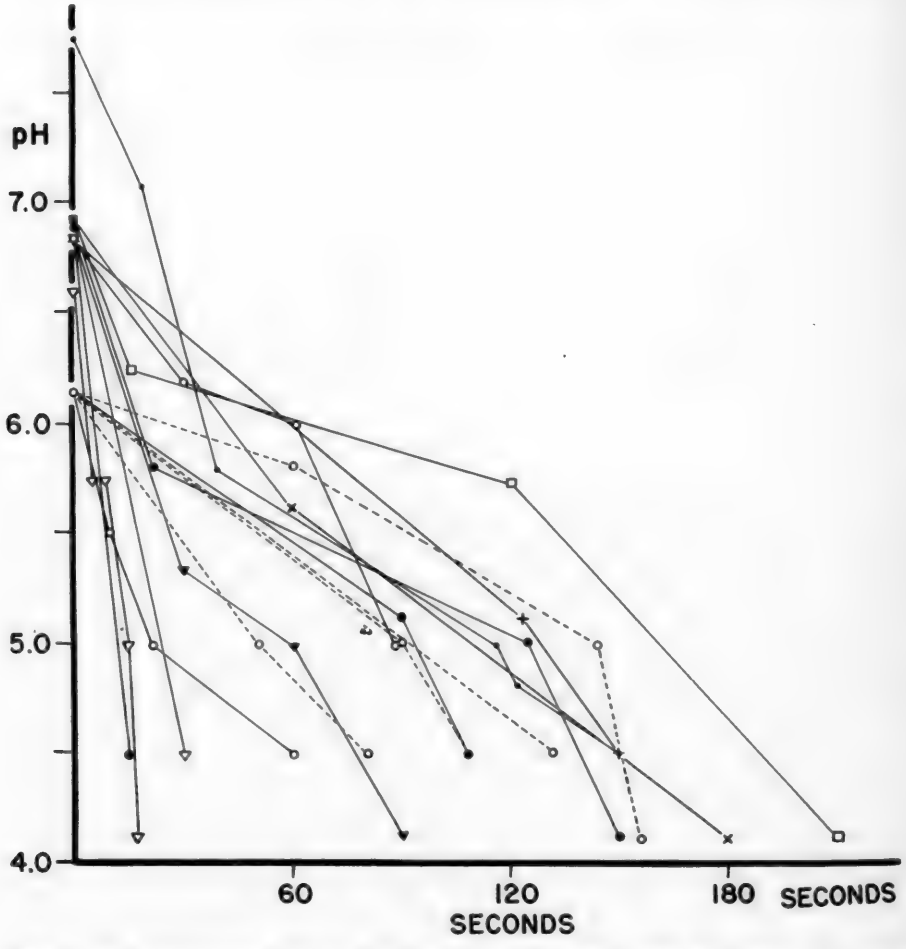
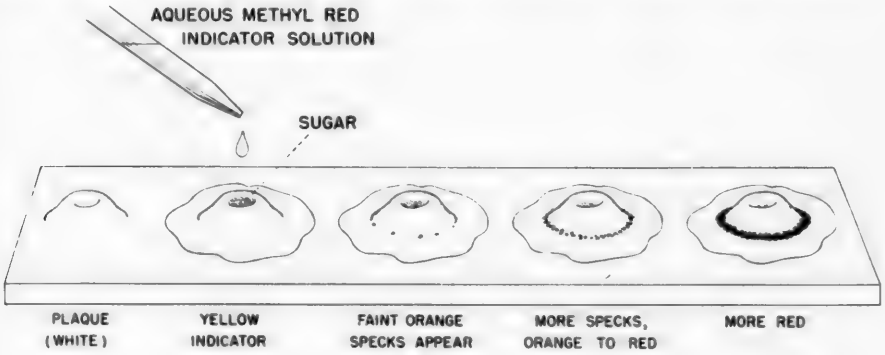


Fig. 1.—Acid development resulting from the action of human plaque on sugar. (15 cases selected to show the variation experienced in the tested group.)

of demineralizing minute areas of enamel in a matter of seconds. In areas where such acid formations are not quickly neutralized or removed by saliva (such as within the interproximal spaces where carious lesions are so frequently seen), repeated episodes would be expected to expand the minute, demineralized areas until these become large enough to be seen grossly as early carious lesions.

ACID PRODUCTION WITH SALIVARY SEDIMENT AND SUGAR

The subjects used in this study were taken from the same student group that served as subjects in the previous plaque study. The study differs from the earlier one however, in that salivary sediment instead of plaque was combined with sugar in the presence of methyl red indicator according to the following steps: (1) Subjects were instructed to dislodge and rid their mouths of such debris as could be readily detected with the tongue for a period of 2 minutes. (2) Each subject then rinsed his mouth vigorously with 30 c.c. of normal saline solution for 1 minute, and collected the rinsings in a clean beaker.



pH	6.3+	5	4.3	4.3−
SECONDS AFTER ADDING SUGAR	0 (CONTROL)	5-15	10-45	20-180

Fig. 2.—Stages in acid development produced by plaque (or salivary sediment) plus sugar.

(3) The rinsings were then divided between two 15 c.c. centrifuge tubes, balanced, and centrifuged at approximately 1,500 r.p.m. for 15 minutes. (4) The clear, supernatant fractions were then discarded; and specimens of the salivary sediment were transferred to form two ½ inch "rings" about 2 inches apart on a clean white tile. (5) Two drops of 0.1 per cent aqueous methyl red indicator solution were then flowed over each ring. (The appearance of orange or reddish specks of color, indicative of acid formation prior to the addition of sugar, disqualified the results obtained with these subjects from the study.) (6) One hundred milligrams of sucrose (or dextrose) were then placed in 1 ring, and the exact clock time (zero time) noted. The second "ring" and indicator were left unsugared to serve as basis for comparison (controls). (7) The appearance of orange and reddish color specks indicative of acid formations in minute quantities were noted along with the exact clock times when these appeared—from which data, time intervals for the acid-forming time peculiar to each subject were computed.

RESULTS

Table I presents the time intervals elapsing between the moments when sugar was added to the salivary sediment test rings, and the moments when the

pH 4.75 acid-denoting orange-red spots were observed. In 4 of the tests no acid formations were evident at any time. In 2 of these it will be seen that the unusual situation occurred in individuals with no, or very low, DMF ratings.

TABLE I

TIME NEEDED FOR THE PRODUCTION OF pH 4.75 ACID CONCENTRATIONS RESULTING FROM THE ACTION OF INDIVIDUAL SALIVARY SEDIMENT ON SUGAR

(AVERAGE pH OF SEDIMENT BEFORE ADDING SUGAR = 6.8)

TIME INTERVALS (SECONDS)	SUBJECT'S CARIES EXPERIENCE (DMF)*
8	0.60
10	.64
10	.42
10	.35
10	.38
13	.43
14	.60
27	.14
28	.68
31	.64
35	.39
38	.50
42	.50
45	.60
45	.50
48	.25
50	.68
51	.46
56	.32
74	.39
80	.46
100	.57
104	.43
110	.50
116	.32
139	.25
190	.50
Showed no acid production	.39
	.00
	.57
	.07

*DMF values are expressed in decimal equivalents of the 28 teeth found to be decayed, missing, or filled.

DISCUSSION

When one compares the acid concentrations achieved in the plaque and salivary sediment groups, and the extreme rapidity with which these demineralizing concentrations can be formed from sugars, it would seem that we have an explanation for what transpires to bring about the initial stage of a carious lesion.

No effort was made to accelerate the reactions involved through the use of elevated temperatures.

Question may be raised concerning the strict comparability of the sugar concentrations used in these studies with those likely to be realized in the mouth. Sugars can usually be detected in the mouth for many minutes following the chewing of soft candies, the drinking of sugared beverages, or the eating of

bread, cookies, cakes, etc. In any of these instances the amount of fermentable carbohydrate would be expected to be far in excess of the few crystals (100 mg.) used in these experiments. The concentrations used here are therefore probably much less than those found in everyday life.

Mention has been made of the DMF ratings of the subjects employed in these studies. No attempt has been made to correlate these with the acid production times peculiar to the plaques or sediments of the respective individuals.

SUMMARY AND CONCLUSIONS

1. Plaque or salivary sediment freshly removed from the mouths of healthy young adult males is shown to be capable of producing enamel-demineralizing concentrations of acid(s) of pH 4.3 or under in from 8 to 281 seconds at room temperatures.

2. Two procedures are described which may be of demonstration value in the practice of preventive dentistry.

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THE pH LEVELS ATTAINED BY COMPACT LAYERS OF ORAL MICROORGANISMS IN CONTACT WITH GLUCOSE SOLUTIONS

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IT HAS often been observed that dental plaques can convert soluble carbohydrate to acid rapidly enough to bring the pH of the plaque to a level that might cause solution of tooth enamel (Stralfors,[1] Stephan,[2] and Miller[3]). This pH decrease occurs locally within such dental deposits because of the action of a variety of bacillary and coccal organisms. Most studies of acid production rate of microorganisms have not employed high concentrations of organisms such as are present in dental plaques, and Stephan and Hemmens[4] criticized the traditional methods of measuring acid production for this reason.

Manly[5] reported the construction of a recording pH meter which permits the study of pH attainment of saliva sediment under conditions of controlled temperature, buffer capacity, and carbohydrate concentration. One advantage of the apparatus lies in the fact that material to be tested can be fitted directly to the surface of a glass electrode while another electrode records the pH of the surrounding buffer solution within the cell. The differential between the 2 electrodes can then be interpreted as an index of rate of acid production.

The purpose of this investigation was to study the pH attained at steady state by samples of dental plaques prepared at a standard thickness, placed in contact with a glass electrode, and immersed in a solution and maintained under controlled conditions of temperature, buffer capacity, and carbohydrate concentration. The pH values were to be compared with the pH attained by pure strains of microorganisms isolated from plaques and saliva and prepared in concentrated form by centrifuging. The knowledge obtained would disclose whether there are several organisms or combinations of organisms that are capable of producing a pH drop equivalent to that of dental plaques.

MATERIAL AND METHODS OF ISOLATION OF MICROORGANISMS

Organisms were isolated from dental plaques which had accumulated on teeth for a period of 12 hours to 6 weeks. Plaque samples were removed with a scaler, broken up by shaking with glass beads or by grinding with a tissue homogenizer, diluted in a range of 10^{-2} to 10^{-12}, and plated on a variety of media. Duplicate plates were incubated aerobically and anaerobically (Brewer jar) at 37° C. from 24 hours to 6 days. The microorganisms were isolated from whole saliva by diluting the samples in physiologic saline in the range of 10^{-2} to 10^{-7}, and plating the aliquots on solid media.

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Colonies isolated from blood agar, Sabouraud agar, nutrient and tomato juice agar, glycerol, ascitic fluid–enrichment agar, and corn meal and eosin-methylene blue agar, were gram-stained, transferred to 10 ml. of dextrose broth (Difco), and replated to observe possible contamination. Isolated strains were identified by means of biochemical tests (Bergey's Manual of Determinative Bacteriology). Streptococci were tested against sera of established Lancefield groups. Members of the "viridans" group were classified according to Kraus, Casey, and Johnson,[6] and lactobacilli according to Rogosa, Wiseman, Mitchell, Disraely, and Beaman.[7]

Dental plaques for study were obtained in the manner mentioned previously from the lingual or labial surfaces of upper and lower anterior teeth of adults. One-hundredth milliliter of this material was drawn into a special Pasteur pipette, deposited into a fitted nylon thimble, and pulled carefully against a Beckman 290-1 microelectrode.

Synthetic plaques were prepared from pure strains of microorganisms. The bacteria were grown in 500 ml. dextrose broth (Difco) for 24 to 48 hours. After centrifugation at 2,000 r.p.m. for 20 minutes, the cell precipitate was washed once with saline buffer at pH 7, once with distilled water, and recentrifuged for 20 minutes at 3,000 r.p.m. With the aid of a capillary pipette, 0.03 ml. of bacterial suspension was deposited in a nylon thimble and fitted gently over the tip of a No. 4990-29 glass electrode having a 7 mm. ball. The electrode was then placed in a Wolff bottle filled with 30 ml. of 0.01 sodium bicarbonate buffer which contained 0.1 M glucose.

The 7 mm. electrodes were used until 3 mm. electrodes could be obtained. A comparison was made between the pH readings of bacterial suspension on the large and small electrodes. There were 8 tests run on the same bacterial suspension, 4 on each type of electrode. The mean differential for the large electrode was 3.00, and for the smaller, 2.93 pH units. For the quantity of material being studied, the 2 electrodes seem to give identical steady-state values, partly because less suspension was employed for the smaller electrode.

The dry weight of samples of plaque and sediment were determined by pipetting 0.01 or 0.03 ml. into a tared platinum-weighing boat, drying at 105° C. for 1 hour, and placing in a desiccator. The boat was placed in a weighing bottle and the weight determined to 0.01 mg. on a semimicro balance 1 hour and 24 hours later.

RESULTS

The information in Table I is concerned with the pH differential at steady state for 20 samples of dental plaques in equilibrium with 0.02 M carbonate buffer. The differential ranged from 1.05 units to 3.03 units, which indicates a threefold variation in differentials among different subjects. This variation could arise either from an increased rate of acid formation or decreased rate of diffusion of buffer or hydrogen ion through the dental plaque.

The data in Table I are grouped to disclose the differentials produced by plaques obtained from upper and from lower teeth. The average drop under controlled conditions is nearly the same. This finding is in contrast with results obtained in vivo. It was reported by Stephan,[8] from pH measurements in vivo, that the pH drop of plaques on labial surfaces of upper teeth, after a sugar rinse, is greater than that of plaques measured on the surfaces of lower teeth. This finding was substantiated for the group of subjects included in Table I by Emerson and Yurkstas.[9] The greater pH drop on upper teeth in vivo cannot be attributable to greater acid formation rate or decreased diffusion rate since under controlled conditions the activity appears to be the same.

TABLE I

ACID PRODUCTION OF PLAQUE MATERIAL FROM UPPER AND LOWER LABIAL AND LINGUAL SURFACES OF PERMANENT TEETH

SUBJECTS CODE LETTER		pH UPPER PLAQUE		pH LOWER PLAQUE	
A		1.05		1.26	
B		1.50		1.70	
C		1.65		1.75	
D		1.70		1.55	
E		1.80		1.67	
F		1.82		2.00	
G		1.87		2.01	
H		1.90		1.20	
I		1.90		2.30	
J		2.07		2.05	
K		2.10		1.73	
L		2.15		2.10	
M		2.20		2.19	
N		2.25		2.11	
O		2.25		2.05	
P		2.39		2.25	
Q		2.50		1.85	
R		2.58		2.65	
S		3.00		2.65	
T		3.03		2.99	
Total	20	Average	2.09	Average	2.00

A few values were obtained on plaques from the same persons at different periods of accumulation. These are presented in Table II. The mean is 2.00 pH units for plaques 24 hours old, and 1.73 units for plaques 96 hours old. The period of accumulation does not appear to have much influence on specific activity.

TABLE II

EFFECT OF PERIOD OF ACCUMULATION ON ACID PRODUCTION OF PLAQUE MATERIAL

SUBJECTS	FIRST READING		SECOND READING	
	pH	AGE	pH	AGE
I	2.06	1 day	2.00	3 days
II	1.94	1 day	1.58	2 days
III	1.79	2 days	1.70	3 days
IV	2.71	2 days	2.10	3 days
V	2.19	3 days	1.71	4 days
VI	1.90	3 days	1.75	4 days

Forty gram-stained smears were prepared from samples of plaques and examined microscopically. Gram-positive and gram-negative cocci, spirochetes, gram-negative and gram-positive rod forms, and gram-negative and gram-filamentous organisms were common to all samples examined.

Some of the microorganisms which were isolated and identified from plaque material are represented in Fig. 1. All the species were tested for ability to produce acid in a concentrated mass. Members of the family Lactobacteriaciae were found to be capable of producing the greatest hydrogen-ion concentration.

Fig. 1.—pH drops of pure strains of oral isolates, oral lactobacilli, and streptococci are capable of producing the greatest pH drops.

The mean differential from 26 tests on *Lactobacillus casei* was 3.8 pH units, and the range was 3.4 to 4.1. The mean and range for *L. plantarum* was slightly lower. The lactobacilli, however, occurred irregularly, whereas the alpha hemolytic and nonhemolytic streptococci were recovered consistently from all plaques. The majority of the streptococci proved capable of producing a pH differential of 3.5 units which produced a terminal pH of 4.5. Five other isolates were less capable of producing a pH decrease, even though they had acid production capacities nearly equal to that of natural plaque material.

Three isolates were less active than natural plaques. Apparently there are several strains of organisms commonly occurring in dental plaques, any one of which would produce a sufficient pH drop to account for the normal reaction of the dental plaque in contact with sugar solution.

The synthetic plaques obtained from strains of organisms were compacted by centrifugation, whereas the natural dental plaque is condensed by some other means. Perhaps the synthetic plaques had a greater solid content, which would bring about a higher acid formation rate per unit volume and perhaps a lesser rate of diffusion. Either of these conditions would tend to produce a lower terminal pH reading. The question of solid content was checked by a determination of the dry weight of representative samples of both groups. Four

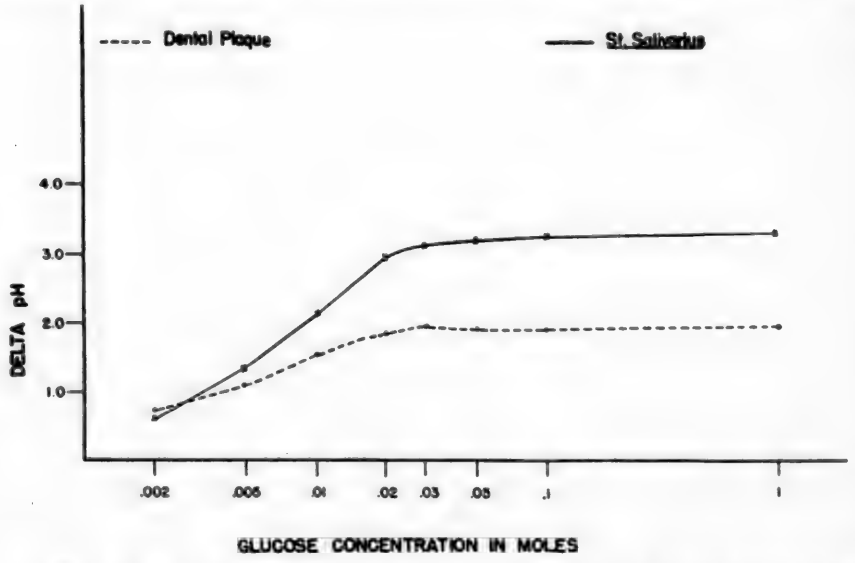

Fig. 2.—Effect of glucose concentration on pH differential of dental plaque and *Streptococcus salivarius*. Precipitates of streptococcal cells give greater pH drops than the same quantity of dental plaque. Glycolysis continues at low concentrations of glucose.

samples of plaque material having 0.01 ml. volume was found to average 1.05 mg. dry weight, corresponding to a solid content of 10.5 per cent. Quadruplicate tests on *Streptococcus mitis* concentrates disclosed a mean solid content of 12 per cent. These findings indicate that the synthetic and natural plaques do not differ appreciably in solid content, which suggests that the differences in terminal pH are probably attributable to differing rates of acid production.

It seemed of interest to compare natural dental plaques and a synthetic plaque prepared from a pure strain of organisms with regard to their ability to produce acid at low substrate concentrations. The dotted line in Fig. 2 represents dental plaque, and the solid line a *Str. salivarius* concentrate. Both were tried in solutions containing glucose ranging from 0.002 M to 1 M. The concentration of glucose is without influence on pH differentials over the range

of 0.03 to 1 M. This holds equally for dental plaque and *Str. salivarius* sediment. From 0.02 M to 0.002 M the differentials are proportional to glucose concentrations. The similarities between differentials of dental plaque and *Str. salivarius* concentrate at 0.002 M glucose, and the points of inflection of the curves, suggest qualitative resemblance between the two materials.

DISCUSSION

One finding of this study is that small amounts of plaque material can produce significant pH differentials when the plaque is in contact with a sugar solution that contains buffer. Yet dental plaque does not possess an acid formation rate which is superior to that of its bacterial components. Any of several microorganisms in plaque prepared in concentrated form and placed in glucose solution, can reach or exceed the pH levels attained by natural plaques. When the level of acid formation is limited by insufficient substrate, dental plaque is equal to a bacterial concentrate in producing a pH differential. This finding suggests that bacterial concentrates do not differ qualitatively from natural plaques in their behavior but have an advantage over natural plaques in possessing less extraneous, inactive substance which dilutes the active cells present.

One of our problems is how to evaluate whether a synthetic plaque matches natural plaque by functional criteria. These findings indicate that the pH differential alone is not definitive. There are several organisms (and potentially many combinations) that are capable of producing hydrogen-ion concentrations with sugar equivalent to or greater than that of natural plaque. The selection of a single pure strain or mixtures of pure strains for exact duplication of the dental plaque requires additional criteria, such as sensitivity to inhibitors or ability to use various substrates.

The acid-producing streptococci can be considered to play the primary role in the pH drop of dental plaque, since they occur constantly in high numbers, and possess ability to form acid rapidly. If this generalization is valid, plaques containing no lactobacilli should attain the same pH as those which contain lactobacilli. Fourteen plaques were examined for the presence of lactobacilli and 10 contained these microorganisms in counts of 35 to 810,000 cells per milliliter. Of the 10 types of lactobacilli isolated, however, only 7 were capable of producing a pH differential greater than 1.5 units. The mean pH differential of the 4 plaques possessing no lactobacilli equaled 1.94 units, and the 3 plaques containing "inactive" strains 1.79 units, while that of the 7 containing "active" lactobacilli equaled 1.85 units. This does not suggest that the lactobacilli aided the plaque in producing a low pH. The relation may be an effect rather than a cause. Perhaps lactobacilli do not contribute appreciably to the maximum rate of acid formation, but their presence indicates conditions favoring their growth—low pH of the plaque during its period of accumulation.

These findings indicate also that the hydrogen-ion concentrations of plaques in the mouth are not always an indication of the specific activity of the plaque

in vivo. When conditions are properly controlled, and the thickness is maintained at a certain value, plaques collected from maxillary and mandibular teeth show the same pH differential whereas the values differ considerably when measured in vivo. Probably upper plaques are thicker than lower or the lower plaques are in contact with liquid having higher buffer capacity.

SUMMARY

The pH differential produced by samples of dental plaques and by synthetic plaques prepared from pure strains of oral microorganisms have been determined under controlled conditions of thickness, buffer capacity, sugar concentration, temperature, and pH of surrounding solution. Natural dental plaque material has been found capable of producing pH differentials of approximately two units. Larger differentials can be attained by some oral lactobacilli and streptococci. Equivalent differentials were attained by 5 other isolates. The specific acid production rate of plaques obtained from upper and lower teeth was found to be the same in contrast with the in vivo findings of other investigators. The results suggest that streptococci play a predominant role in acid formation rate and that lactobacilli have a minor part because of their less frequent occurrence and lower numbers.

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THE FERMENTATION OF SORBITOL BY CERTAIN ACIDOGENIC ORAL MICROORGANISMS

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SORBITOL possesses certain properties such as sweetness and humectant action that would make it a desirable constituent of oral preparations. The question of whether it would be fermented by oral microorganisms and hence contribute to caries, however, arises. Grubb[1] conducted studies along this line and, in general, arrived at the conclusion that it would be favorable, compared with glucose and sucrose, since it was fermented more slowly than these sugars when it was fermented at all. Our studies have been designed to obtain a more definite answer to the question of sorbitol fermentation by oral microorganisms both by pure culture and the mixed flora of saliva and to determine certain factors in this fermentation that would be pertinent to the situation in the oral cavity.

MATERIALS

The pure cultures employed in this investigation were freshly isolated from saliva of caries-susceptible individuals, unless otherwise noted. Since the saliva was obtained by paraffin stimulation, the isolated organisms should also include some from the dental plaque. The lactobacilli were isolated on tomato juice agar at pH 5 and designated "rough" and "smooth" according to their colonial morphology. These designations probably correspond generally to *Lactobacillus acidophilus* and *L. casei*, respectively, as described by Rogosa, Wiseman, Mitchell, and Disraeley.[2]

The chemicals employed in this investigation have all been C.P. grade. "Sorbo," commercial grade sorbitol (manufactured by The Atlas Powder Company), was also tested and the results with this product are entirely comparable to those with C.P. sorbitol.

The methods employed in this investigation are standard and need not be described in detail. In the experimental section will be found brief descriptions of how these methods were employed in the several aspects of the study.

EXPERIMENTAL

Pure Culture Studies.—One of the first problems encountered in this study was finding a carbohydrate-free medium which would support the growth of the oral bacteria under investigation. In an attempt to find such a medium,

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a preliminary study of 7 lactobacillus, 6 streptococcus, and 3 staphylococcus cultures was conducted. The cultures were maintained in brain-heart infusion broth. Three loops of each culture were inoculated into 5 ml. of 2 per cent heart infusion broth and into 2 per cent trypticase broth containing 0.1 per cent yeast extract with and without 1 per cent glucose, glycerol, and sorbitol. Bromcresol purple at a concentration of 0.2 per cent was used as an indicator for acid production. Examinations of the cultures were made at 24 and 48 hours.

Of the 7 lactobacilli, 2 were described as "rough" forms on the basis of their colonial morphology when grown on tomato juice agar at pH 5.0. The other 5 were described as "smooth" strains which presented a smooth, glistening type of growth. It had been previously observed that the so-called "rough" strains grew with difficulty in tomato juice broth.

On comparing the 2 test media for support of growth and acid production, trypticase yeast extract broth appeared to be superior. In addition, it was observed that maximal growth and acid production were obtained in broth containing glucose. Growth in media containing sorbitol or glycerol or no added carbohydrate was considerably slower. The results for acid production in the presence of the various substrates are shown in Table I. In the 2 cases where sorbitol was fermented by the lactobacilli, it was not indicative of strong acid production. The 2 "rough" strains of lactobacilli did not grow, and hence no production of acid was observed.

TABLE I
FERMENTATION REACTIONS OF ORAL MICROORGANISMS

TYPE OF ORGANISMS	NUMBER TESTED	NUMBER FERMENTING GLUCOSE	NUMBER FERMENTING GLYCEROL	NUMBER FERMENTING SORBITOL
Streptococci	6	6	3	2
Lactobacilli (smooth)	5	5	0	2
Lactobacilli (rough)	2	0*	0*	0*
Staphylococci	3	3	2	0

*No growth.

Inasmuch as unmodified tomato juice broth (TJB) contained a considerable amount of fermentable carbohydrate, it was not usable as a test medium. It was necessary to remove most of the carbohydrate from the tomato juice (TJ) by inoculating it with yeast cells and incubating for 24 hours. Media containing 40 per cent fermented tomato juice, 2 per cent trypticase, 0.1 per cent yeast extract, 1 per cent peptone, and 5 mg. of malate, citrate, alpha-ketoglutarate, and the sodium salt of ethyl oxalacetate were added per tube. Glycerol, glucose, and sorbitol were the test carbohydrates.

Fermentation of the glucose and sorbitol by the "smooth" strains was more rapid than in previous observations. "Rough" strains of the lactobacilli grew very little if any. Glycerol was attacked by only 2 streptococci and none of the lactobacilli. Further attempts to develop a medium which would give consistent growth and acid production of both "rough" and "smooth" forms of the lactobacilli were made by separately adding various

organic compounds. Among these were adenosine triphosphate, adenylic acid, magnesium hexose diphosphate, lactose, maltose, and sucrose. None of these compounds stimulated the growth of the "rough" strains.

It was finally found that by adding Tween 80 at a concentration of 0.05 per cent to the medium containing 40 per cent fermented tomato juice, 2 per cent trypticase, 0.1 per cent yeast extract, 1 per cent peptone, and the acids of the tricarboxyllic acid cycle previously used, we could obtain good growth of the "rough" strains. Rogosa and Mitchell[3] had previously reported on the beneficial effect of Tween 80.

With the development of such a medium, we were able to run fermentation tests on all the lactobacilli. Thirty lactobacillus cultures were used in these tests. Of the 30 cultures, 11 were of the "rough" variety. Six of the "smooth" organisms were from caries-immune individuals.* Only one "rough" organism from caries-immune individuals was studied. All the rest of the organisms studied were from susceptible individuals. The "smooth" cultures were maintained in untreated TJB while the "rough" strains were maintained in untreated TJB with 0.05 per cent Tween 80. These cultures were tested for their ability to ferment glucose, glycerol, and sorbitol at a concentration of 1 per cent. The media were distributed in tubes containing 5 ml. each. The media were inoculated with approximately 0.1 ml. of the culture (24 to 48 hours old). The results of the fermentation test are shown in Table II for glucose and sorbitol as substrates. Glycerol is not included in the results, since none of the lactobacilli attacked it.

TABLE II

FERMENTATION OF GLUCOSE AND SORBITOL IN 48 HOURS BY "SMOOTH" AND "ROUGH" STRAINS OF LACTOBACILLI FROM CARIES-SUSCEPTIBLE AND CARIES-IMMUNE INDIVIDUALS

SOURCE	TYPE OF CULTURE	NUMBER TESTED	NUMBER FERMENTING GLUCOSE	NUMBER FERMENTING SORBITOL
Cares susceptible	Smooth	13	13	10
Caries susceptible	Rough	10	10	0
Caries immune	Smooth	6	6	3
Caries immune	Rough	1	1	0

It was found that none of the rough strains fermented sorbitol, and that they fermented glucose only very weakly.

Similar results were later obtained employing the medium described by Rogosa, Wiseman, Mitchell, and Disraeley,[2] but with the potassium dihydrogen phosphate and sodium acetate omitted.

Studies of pH were carried out in an attempt to measure possible differences between strains in rates and amounts of acid production with the various substrates. Tubes containing 10 ml. of each type of media previously used in the fermentation tests were set up so that pH measurements could be taken at intervals of 3, 6, 12, 24, 36, and 48 hours. The media were adjusted

*In this study an adult free of caries and fillings and having no missing teeth was considered as caries-immune.

to an initial pH of 7.0. The inoculum consisted of 1 ml. of 24 to 36 hour culture of "smooth" organisms grown in TJB or "rough" grown in TJB with Tween 80 added. A Beckman pH meter was used for readings.

Preliminary pH studies carried out with "smooth" strains of lactobacilli and streptococci in broth containing 0.2 per cent trypticase and 0.1 per cent yeast extract had shown that the pH of glucose reached 5.0 with the lactobacilli in 12 to 24 hours and with the streptococci in 6 hours. Sorbitol was fermented much slower than glucose. Streptococci fermenting sorbitol produced a pH of 5.0 in 12 hours and the lactobacilli gave evidence of very little change during this time period (see Table III).

TABLE III

CHANGES IN pH WITH REPRESENTATIVE STREPTOCOCCI AND LACTOBACILLI (SMOOTH TYPE)

	ADDITIVE TO BASAL MEDIUM	TIME IN HOURS						
		0	3	6	12	24	36	48
Lactobacillus No. 2	———	7.0	7.0	7.0	7.0	6.8	6.9	7.1
	glucose	6.9	6.9	6.8	6.6	4.4	4.3	4.5
	glycerol	7.0	7.1	7.0	7.1	6.8	7.0	7.2
	sorbitol	7.0	7.1	7.0	6.7	6.3	5.4	4.9
Lactobacillus No. 3	———	7.0	7.0	6.8	7.0	7.4	7.5	7.5
	glucose	6.9	6.8	6.6	5.9	5.1	5.2	5.2
	glycerol	7.1	7.0	6.9	7.0	7.4	7.4	7.5
	sorbitol	7.0	7.0	6.8	7.0	7.2	7.5	7.5
Lactobacillus No. 17	———	7.0	7.1	7.1	7.0	6.9	7.0	7.3
	glucose	6.9	6.9	6.8	6.4	4.8	4.6	4.7
	glycerol	7.0	6.9	7.1	7.0	6.9	7.1	7.2
	sorbitol	7.0	6.9	7.0	7.0	7.0	7.0	6.7
Streptococcus No. 1	———	7.0	6.8	6.7	6.8	6.8	7.2	7.4
	glucose	6.9	6.5	5.4	5.0	4.6	4.6	4.8
	glycerol	7.0	6.8	6.4	5.7	5.1	5.2	5.5
	sorbitol	7.0	6.7	6.5	5.6	5.0	5.0	5.0
Streptococcus No. 14	———	7.0	6.9	6.9	6.8	6.8	6.9	7.3
	glucose	6.9	6.9	6.7	6.0	4.9	5.0	5.1
	glycerol	7.0	7.0	6.9	6.7	6.4	6.3	6.4
	sorbitol	7.0	6.9	6.9	6.8	7.0	7.0	7.3
Streptococcus No. 19	———	7.4	7.3	7.5	7.5	7.5	—	—
	glucose	7.1	6.5	4.9	4.6	4.5	—	—
	glycerol	7.4	7.3	7.2	7.0	7.1	—	—
	sorbitol	7.3	7.0	5.8	5.0	4.9	—	—

Since the results of other studies by Smith[5] suggested that fructose might be readily metabolized by the "rough" strains, we included it as a test substrate. Table IV shows the pH produced by the fermentation of glucose and sorbitol by "smooth" and "rough" strains and of fructose by the "rough strains." It was observed that the fermentation of glucose appeared to be somewhat slower in this medium than in the medium containing trypticase and yeast extract. The difference was probably due to the greater buffering power of the medium.

Serial transfers of cultures of lactobacilli in the presence of sorbitol failed to yield organisms capable of greater acid production from sorbitol. Those not fermenting sorbitol still failed to do so after 8 transfers. These results,

together with similar negative results by Thunberg and manometric techniques, demonstrated that those lactobacilli failing to ferment sorbitol apparently were genetically incapable of such action and did not readily acquire such ability.

TABLE IV. DISTRIBUTION OF "ROUGH" AND "SMOOTH" STRAINS OF LACTOBACILLI BY pH PRODUCED WITH GLUCOSE, SORBITOL, AND FRUCTOSE IN 48 HOURS

TYPE OF CULTURE	NUMBER OF STRAINS TESTED	GLUCOSE		SORBITOL		FRUCTOSE	
		pH 5.0 OR BELOW	ABOVE pH 5.0	pH 5.0 OR BELOW	ABOVE pH 5.0	pH 5.0 OR BELOW	pH 5.1 TO 5.7
Rough	11	4	7*	0	11‡	4	7
Smooth	19	17	2†	5	14	§	§

*pH values stopped at 6.7 or higher.
†Lowest pH, 5.1.
‡No appreciable activity.
§No test.

Saliva Studies.—In order to obtain some information on the influences of sorbitol on the mixed flora of the mouth, sorbitol and other materials were added to fresh saliva. Changes in pH and in flora were followed; in addition, transfers to similar saliva media were made and the same changes observed. Very similar results were noted in the 14 saliva samples employed so the results of the pH studies are recorded as averages in Table V.

TABLE V

AVERAGE pH CHANGES IN SALIVA WITH ADDED FERMENTABLE MATERIALS

HOURS INCUBATION	GLUCOSE 1%	SORBITOL 1%	GLYCEROL 1%
	Initial Samples		
12	4.8	6.9	7.1
24	4.6	7.0	7.6
	First Transfer		
12	4.2	6.2	7.0
24	4.2	6.3	7.6
36	3.9	5.7	7.9
	Second Transfer		
6	5.5	6.4	7.6
24	4.2	5.4	7.6
48	3.6	5.7	7.7*

*At this time 2 samples demonstrated glycerol fermentation; one sample showed a pH of 5.1 and another 6.2, indicating the development of a glycerol-fermenting population in these salivas.

Microscopic examination of the saliva samples showed the following selective actions of the added compounds: (1) Glucose plus saliva favored the growth of streptococci early in the incubation period followed, after 24 hours, by growth of lactobacilli. Streptococci rapidly disappear after 24 hours. (2) Glycerol favored the development of gram-negative rods. The odor of each tube as well as the pH changes were indicative of proteolysis. (3) Microscopic examination of the 14 samples containing sorbitol after 48 hours' incubation of the second transfer showed large numbers of gram-negative rods, gram-negative cocci and, in most samples, gram-positive rods and cocci.

Results of a series of 3 successive transfers in sorbitol-saliva in another experiment also showed a predominance of gram-negative rods. When a 1:1,000 dilution was streaked on plates containing sorbitol, only gram-negative rods could be isolated. They were sorbitol fermenters. Further incubation of the plates resulted in a basic reaction demonstrating that these organisms are also proteolytic.

In view of the beneficial effects of certain surface-active agents on the growth of some oral lactobacilli, saliva samples containing the above materials plus Tween 80 or Triton A-20 were compared with those not containing these agents. No significant differences were noted.

Metabolic Studies.—In addition to the more or less direct tests reported above, detailed studies on the metabolism of sorbitol by both cells and cell-free extracts of a "smooth" strain of lactobacillus yielded certain relevant information. While a complete report on these experiments will be presented, we should note here certain of the results obtained.

Sorbitol was shown to be attacked by an adaptive enzyme or enzymes. This was demonstrated by growing cells in the presence of either sorbitol or glucose and then testing washed suspensions of these cells for acid production. In the results of such an experiment reported in Table VI, acid production was followed by potentiometric pH determinations with heavy suspensions of cells, standardized turbidimetrically with a Beckman Model B spectrophotometer.

TABLE VI

ACID PRODUCTION BY GLUCOSE AND SORBITOL GROWN CELLS

SUGAR OR ALCOHOL IN GROWTH MEDIUM	SUGAR OR ALCOHOL TESTED WITH CELL SUSPENSION	pH AT TIMES (MIN.) INDICATED						
		0	5	10	15	20	25	30
Glucose	Glucose	7.0	6.6	5.8	4.8	4.4	4.2	4.1
	Sorbitol	7.0	6.9	6.9	6.8	6.7	6.65	6.6
		7.0	7.0	7.0	7.0	7.0	7.0	7.0
Sorbitol	Glucose	7.2	6.6	5.5	4.7	4.2	–	4.1
	Sorbitol	7.2	7.0	6.8	6.6	6.3	–	5.9
		7.2	7.2	7.2	7.2	7.2	–	7.1

These results, in addition to demonstrating the adaptive nature of the enzyme or enzymes acting on sorbitol, further demonstrate the slower rate of acid production from sorbitol. It should also be pointed out that the pH changes described here with cell suspensions are not dependent upon growth as they were in previous tests of fermentability. Thus, the slow rate of acid production reported above in growing cultures was not due to the inability of the cells to grow rapidly.

Demonstration of the adaptive nature of the enzyme or enzymes attacking sorbitol has certain consequences that are associated with the phenomenon of adaptive enzyme production. One of these involves the competition that is commonly directed against the production of an adaptive enzyme by other substrates. In this case, one might expect that glucose would inhibit the production of the enzyme or enzymes active on sorbitol. The results in Table VII demonstrate this competitive effect of glucose, by showing that in the

presence of small amounts of glucose, the glucose is first utilized, then adaptation, and growth begins with the sorbitol present. The experiment consisted of following acid production during growth in media containing 1 per cent sorbitol plus small amounts of glucose.

TABLE VII

ACID PRODUCTION WITH SORBITOL AND GLUCOSE BOTH PRESENT

	pH AT TIMES (HOURS) INDICATED											
	0	3	4.5	6	7	8.5	9.5	10.5	12	14	16	18
1% Sorbitol*	6.9	6.7	6.6	6.5	6.45	6.5	6.5	6.4	6.3	6.15	5.8	5.55
1% Sorbitol + 0.05% glucose	6.9	6.7	6.6	6.4	6.0	6.0	6.0	5.95	5.9	5.75	5.6	5.3
1% Sorbitol + 0.01% glucose	6.9	6.7	6.55	6.3	5.75	5.6	5.6	5.6	5.55	5.45	5.3	5.15

*A small amount of glucose, probably 0.01 per cent, was introduced with the inoculum.

It will be seen that after an initial rapid drop in the pH proportional to the amount of glucose present, a period of 3 to 4 hours followed in which no further acid production was detected. This inactive period demonstrates that the cells had not adapted to sorbitol while glucose was present. After this period further decreases in pH demonstrate adaptation to the sorbitol.

In examining acid production from sorbitol and glucose manometrically, by release of CO_2 from bicarbonate buffer,[4] it was shown that only about 1 micromole of acid (21 μl CO_2 observed; 22.4 μl theoretical value) was produced per micromole of sorbitol while approximately 2 micromoles (40 μl CO_2 observed; 44.8 μl theoretical value) were produced per mole of glucose or fructose. This denotes the production of 1 micromole of a nonacid product and is consistent with hydrogen balance requirements since sorbitol is more reduced than the hexoses. This nonacid product is undoubtedly glycerol, and its production can be responsible for the significantly lower rate of acid production from sorbitol. These results also indicate that this strain of lactobacillus has a homolactic type of fermentation, as would be anticipated.

Aerobically, it has been demonstrated manometrically (O_2 uptake) that the optimum pH for sorbitol oxidation lies between pH 7 and 8, with a rapid decrease in activity on either side of this optimum.

Although we have not investigated the metabolic activities of the streptococci as we have the lactobacilli, it would be expected that they behave in very much the same manner as the lactobacilli because of their general physiologic similarities.

SUMMARY AND CONCLUSIONS

On the basis of the results outlined above, coupled with knowledge of bacterial physiology and the caries problem, it is our conclusion that the use of sorbitol would not contribute significantly to acid production and, thereby, to dental caries. It is even possible that benefits might arise from its use.

This conclusion is based on the following: (1) Only some strains of the various species studied ferment sorbitol. None of the rough strains of lacto-

bacilli nor staphylococci tested fermented sorbitol. (2) With strains fermenting sorbitol, acid production was slow, the quantity small, and conditions must be optimal. These conditions include a near neutral pH, continued presence of sorbitol, and virtual absence of glucose. (3) In the mixed flora of saliva, sorbitol leads to selection of gram-negative organisms, not the acidogenic cocci and gram-positive rods, and the pH believed necessary for decalcification is not reached. (4) Ability to ferment sorbitol is not readily acquired and there appear to be certain theoretical limits to the amount of acid that can be produced from sorbitol.

The authors wish to thank Dr. Gordon Green for the isolation of some of the organisms employed in this study.

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DIMETHYLAMINOBENZALDEHYDE AS A MEASURE FOR SALIVARY "TRYPTOPHANE"

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METHODS in common use for the measurement of salivary "tryptophane" have usually been adaptations of methods previously applied to foods. Among others, the following 2 tests have been used. (1) Horn and Jones[1]: Dimethylaminobenzaldehyde in 11N HCl and 0.2 per cent sodium nitrite. (2) Shaw and McFarlane's adaptation of the Hopkins-Cole technique[2]: Glyoxylic and sulfuric acids. Shaw and McFarlane contrasted the p-dimethylaminobenzaldehyde and glyoxylic acid methodologies for testing the tryptophane content of foods. They concluded that the former gives erroneous results and confirmed the reliability of the Hopkins-Cole[3] technic.

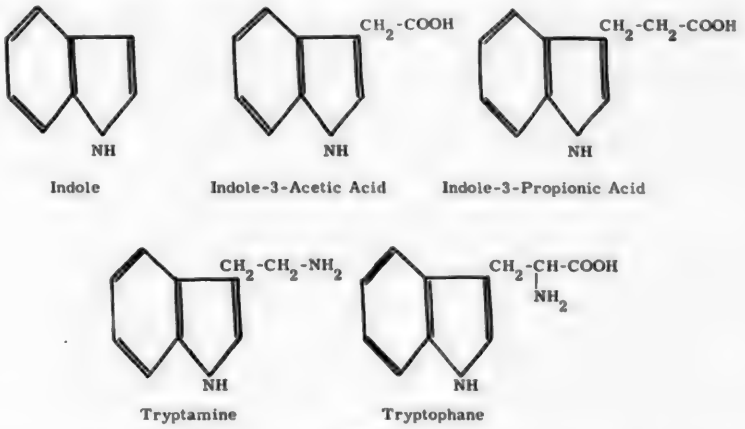

Fig. 1.

Since saliva has in it not only tryptophane and indole, but also indole acetic acid[4] and possibly other indole derivatives,[5] it seemed desirable to find out whether the p-dimethylaminobenzaldehyde methodology distinguishes certain indole structures from each other, and what results might be obtained by testing saliva to which these indole compounds had been added. As is quite obvious, the indole ring is a part of each of these molecules. Other related compounds, not subjected to test in this study, include skatole and indole butyric acid.

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METHOD AND MATERIAL

In the tests reported below the method described by Stack[6] was followed: To 1.5 ml. of solution to be tested add 0.5 ml. of a 5 per cent solution of dimethylaminobenzaldehyde in 11N HCl; add 5 ml. of 11N HCl; after 5 minutes add 0.05 ml. 0.2 per cent sodium nitrite; mix well and read after 10 minutes on a Photelometer, using red filter. This test was applied to the following materials made up as 0.1 per cent solutions: indole, indole-3-acetic acid, indole-3-propionic acid, tryptamine [3-(2-aminoethyl) indole hydrochloride], and dl-tryptophane.

RESULTS

Results are presented in Table I for 0.1 per cent solutions and their dilution by 1/2, 1/4, 1/8, and 1/16. After treatment with the reagents indicated above, the colors obtained were read using a Photelometer with a red filter.

TABLE I

PHOTELOMETER READINGS

SUBSTANCE	AT 0.1% LEVEL	AT DILUTIONS OF 0.1% BY			
		1/2	1/4	1/8	1/16
Indole	2	2	5	13	30
Indole-3-acetic acid	4	5	7	13	27
Indole-3-propionic acid	3	5	10	20	37
Tryptamine	3	6	10	17	32
Tryptophane	4	7	12	19	32

The 5 substances under investigation were also made up to 0.00625 per cent solution and similarly tested. The results obtained are presented in Table II.

TABLE II

SUBSTANCE IN 0.00625% SOLUTION	PHOTELOMETER READING
Indole	24
Indole-3-acetic acid	18
Indole-3-propionic acid	28
Tryptamine	15
Tryptophane	15
Indole, Indole-3-acetic, Tryptophane (0.5 ml. each)	22

From this table it is evident that using the dimethylaminobenzaldehyde and sodium nitrite technic, the Photelometer readings fail to show a clear distinction between these 5 substances. Visually the blue-green colors obtained from these 5 substances appear very similar. The results of applying this test to centrifuged and noncentrifuged saliva to which these respective substances (in 0.00625 per cent solutions) had been added are shown in Table III.

Inspection of Table III reveals that a blanketing effect occurs in saliva upon the addition of any of these 5 substances. Under these conditions the identity of each of these materials tends to be lost.

TABLE III

SUBJECT	MATERIAL	MILLILITER	CENTRIFUGED SALIVA (SUPERNATANT)	CENTRIFUGED SALIVA (SUPERNATANT) 18 HOURS (REREAD)		NONCENTRIFUGED SALIVA (WHOLE) 18 HOURS (REREAD)	
	Subject A		*X*	*Y*	*Y*[1]	*Z*	*Z*[1]
Saliva	(1 ml.) + Water	0.5	46	39	46	32	37
Saliva	(1 ml.) + Indole	0.5	34	36	43	27	33
Saliva	(1 ml.) + Indole-3-acetic	0.5	28	26	35	20	27
Saliva	(1 ml.) + Indole-3-propionic	0.5	33	32	40	24	31
Saliva	(1 ml.) + Tryptamine	0.5	29	24	31	19	25
Saliva	(1 ml.) + Tryptophane	0.5	28	27	32	20	25
	Subject B						
Saliva	(1 ml.) + Water	0.5	50	53	57	46	53
Saliva	(1 ml.) + Indole	0.5	44	45	48	38	44
Saliva	(1 ml.) + Indole-3-acetic	0.5	33	31	41	24	34
Saliva	(1 ml.) + Indole-3-propionic	0.5	36	40	47	30	39
Saliva	(1 ml.) + Tryptamine	0.5	30	30	36	25	32
Saliva	(1 ml.) + Tryptophane	0.5	33	32	37	26	32

X = Photelometer reading of a centrifuged sample.
Y and Z = Photelometer reading of a split sample, centrifuged and noncentrifuged.
Y[1] and Z[1] = Photelometer reading of Y and Z after 18 hours.

DISCUSSION

It is of particular interest to note how closely the figures approximate each other upon the addition of either tryptamine, tryptophane, or indole-3-acetic acid since unpublished studies[4] from this laboratory indicated that there is not a consistent relationship between the amount of dental caries and the amount of indole-3-acetic acid in saliva.

The second method in common use for measuring tryptophane is that of Hopkins and Cole.[3] The chief reagents for this test are glyoxylic acid and concentrated sulfuric acid. Of the 5 substances investigated here, the Hopkins-Cole test differentiated indole-3-acetic acid (yellow). Indole-3-propionic, tryptamine, and tryptophane could be differentiated only in certain dilutions. Indole, per se, either concentrated or dilute, gives (with Hopkins-Cole) a distinctive cherry red or pink coloration. In doing a large number of salivary tests with glyoxylic-sulfuric acids in this laboratory, a certain number of salivas have produced a strong reddish hue which may have been due to indole interference.

Thus, neither this method, used by Turner and Crowell[7] in 1947 to designate "tryptophane or similar indole-containing molecules," nor the one (using p-dimethylaminobenzaldehyde) employed by Stack[6] to designate tryptophane seem sufficiently differentiating when applied to saliva for identifying tryptophane if other indole-containing molecules are present. To do this requires auxiliary methods. Stack's report of a lack of correlation between salivary tryptophane and dental caries experience was based on a test which does not differentiate tryptophane from indole-3-acetic acid or certain other indole-containing molecules.

CONCLUSION

Experiments indicate that the dimethylaminobenzaldehyde test is essentially nondifferentiating for indole, indole-3-acetic acid, indole-3-propionic acid, tryptamine, and tryptophane, when these are added to salivary samples. Therefore, it would appear that this methodology applied to saliva does not provide an adequate means for identifying tryptophane differentially.

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OVERSATURATION OF SALIVA WITH HYDROXYAPATITE

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DENTAL ENAMEL consists of about 90 per cent of hydroxyapatite (HA) of the formula 3 $Ca_3(PO_4)_2.CA(OH)_2$.[1, 2] In vivo enamel is permanently in contact with saliva. It is within this "physico-chemical" system of enamel and saliva, that processes of solution and crystallization are taking place. This study was designed to assess, in vitro, the influence of HA upon the calcium and phosphate content, the pH and the buffering capacity of stimulated and unstimulated saliva.

EXPERIMENTAL METHOD

As described previously,[3] "unstimulated saliva" was collected by allowing saliva to drop into a container from the slightly opened mouth of a mentally diverted patient. "Stimulated saliva" was drained from the mouth of the same person after a piece of rubber was chewed. Only persons who were entirely free from dental caries as well as periodontal disease were used in this experiment. Ten milligrams of pure precipitated HA[4] were added to each 2.5 c.c. of both stimulated and unstimulated saliva and brought to suspension by stirring with a glass rod. After addition of a drop of mercuric chloride the solution was kept at a temperature of 37° C. for 20 hours* while being shaken and stirred frequently. Two cubic centimeters of clear liquid, separated from apatite and opaque saliva by means of a centrifuge, was analyzed for dissolved calcium according to the method of Kramer and Tisdall.[5] The amount of inorganic phosphate in a weighed drop of clear liquid was examined after the method of Beerenblum and Chain.[6] In order to establish the pH of the saliva, 2.0 c.c. of untreated saliva were titrated first with n/20 sodium hydroxide to the changing point of added phenolphthalein (pH = 8.5) and subsequently with n/20 hydrochloric acid to the changing point of added methyl red (pH = 5.5).† Then the amounts of dissolved calcium and phosphate were determined using the methods explained before. Finally, in order to measure the buffering capacity, the amount of n/10 hydrochloric acid needed to alter the pH of 1 c.c. of saliva from 8.5 to 5.5 was established.

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*Previous experiments had shown that solubility balance was established within 4 hours.

†It is impossible to establish the pH of the saliva exactly by means of the chinhydrone electrode, because chinhydrone soon turns brown and is oxidized.

TABLE I

AMOUNTS OF CALCIUM AND PHOSPHATE IN UNSTIMULATED AND STIMULATED SALIVA PRIOR AND SUBSEQUENT TO CONTACT WITH HA, THE pH AND THE BUFFERING CAPACITY OF UNTREATED SALIVA

NAME OF PATIENT	CONTENT OF CALCIUM (MG. PER CENT Ca)			CONTENT OF PHOSPHATE (MG. PER CENT PO_4)				
	BEFORE HA	AFTER HA	DIFFERENCE	BEFORE HA	AFTER HA	DIFFERENCE	pH	c.c. n/10 HCl pH 8.5 5.5
				a. *Unstimulated Saliva*				
P. K.	13.9	13.4	0.5	12.8	10.3	2.5	6.3	0.36
K. H.	7.4	3.0	4.4	11.3	7.1	4.2	6.5	0.24
L. S.	16.8	14.2	2.6	15.2	13.8	1.4	6.8	0.44
H. A.	11.0	6.3	4.7	16.0	12.4	3.6	6.6	0.17
H. K.	8.2	7.9	0.3	12.7	12.4	0.3	5.7	0.32
W. H.	15.9	8.0	7.9	13.1	10.5	2.6	6.1	0.45
E. D.	9.7	5.7	4.0	17.7	13.7	4.0	6.8	0.40
F. G.	14.0	4.4	9.6	11.0	6.8	4.2	6.1	0.17
E. B.	7.8	4.6	2.2	10.6	6.9	3.7	5.8	0.44
L. C.	11.2	8.8	2.4	17.4	10.2	7.2		0.21
	10.9		4.5	14.1		3.3	6.0	0.32
	±0.8		±0.9	±0.8		±0.5	±0.2	±0.04
				b. *Stimulated Saliva*				
P. K.	10.4	4.0	6.4	18.9	14.4	4.5	7.0	0.09
K. H.	8.1	1.2	6.9	16.0	12.6	3.4	6.0	0.17
L. S.	10.8	3.8	7.0	10.3	9.1	1.2	5.9	0.20
H. A.	10.6	2.1	8.5	17.8	6.2	11.2	6.9	0.08
H. K.	14.0	1.7	12.3	10.1	7.5	2.6	6.1	0.13
W. H.	14.2	3.3	10.9	13.9	10.6	3.3	6.4	0.02
E. D.	16.9	14.2	2.7	19.0	8.1	10.9	6.6	0.31
F. G.	9.2	4.0	5.2	19.2	6.8	12.4	6.8	0.24
L. C.	11.0	1.4	9.6	13.2	10.7	2.5	6.5	0.02
	11.8		7.8	15.5		5.8	6.5	0.13
	±0.8		±0.8	±1.1		±1.3	±0.1	±0.03

RESULTS

Table I demonstrates that (1) samples of unstimulated and stimulated saliva show no significant difference (i.e., exceeding the threefold of the probable error of the average) in the Ca– and P– content, (2) samples of unstimulated saliva are more acid (lower pH) and have a stronger buffering capacity (larger consumption of acid from the phenolphthalein change to the methyl red change) than the samples of stimulated saliva, (3) the Ca– and P– concentrations are decreased in both the unstimulated and the stimulated saliva samples following in contact with HA, and (4) the decrease of Ca– and P– concentration subsequent to the 20 hours' contact with HA is smaller in unstimulated saliva than in stimulated saliva.

DISCUSSION

The decrease in the Ca– and P– concentrations of unstimulated and stimulated saliva following contact with HA indicates that both types of saliva represent oversaturated solutions of HA. By addition of pure hydroxyapatite crystals, HA crystallizes until a solubility balance is established. Although unstimulated saliva has the same concentrations of calcium and phosphate, it is

less saturated than stimulated saliva as it is more acid and better buffered. Unstimulated saliva therefore possesses a higher dissolving capacity for HA. The stronger buffering capacity of unstimulated saliva is probably due to the higher content of mucin,[7] which causes a higher viscosity. It was shown to be produced by the sympathicotonic status of the salivary glands during secretion.[3]

The fact that saliva represents an oversaturated solution of HA has found little mention in the literature. Price[8] showed a decreased calcium content and increased phosphate content of saliva when it was shaken with powdered bone containing HA. This decrease in the calcium content seems to indicate that HA was crystallized in the saliva, while the increase of the phosphate content may have derived from small quantities of soluble phosphate of the bone.[9, 10]

Similar phenomena have been reported when powdered enamel was brought into contact with saliva.[11, 12] By shaking saliva with "tricalciumphosphate" Becks,[13] Karshan,[14] and Rae and Clegg[15] observed decreased calcium concentration, as well as increased phosphate concentration. Becks contended that saliva might contain a "negatively loaded, absorbable" calcium compound. However, "tricalciumphosphate" is not stable in the presence of water, and the commercial product "tricalciumphosphate" is a changing mixture of HA and dicalciumphosphate. Hence the findings of increased phosphate content of saliva after mixing with powdered bone, enamel powder, and "tricalciumphosphate" may be reconciled with our observations of decreased phosphate concentrations by the fact shown by Schleede, Schmidt and Kindt[16] that dicalciumphosphate undergoes hydrolyzation into HA and dissolved phosphoric acid:

$$10\ CaHPO_4 + 2\ H_2O = 3\ Ca_3(PO_4)_2.Ca(OH)_2 + 4\ H_3PO_4.$$

The origin of this phosphoric acid also will explain why Rae and Clegg observed an acid reaction when they shook "tricalciumphosphate" with saliva or water. Therefore it is necessary to place saliva under influence of *pure* HA whenever oversaturation of the saliva with HA is to be demonstrated.

The state of oversaturation of the saliva with HA probably has some bearing upon the mechanism of the Fosdick-test[17] which is used for determining caries susceptibility. Stimulated saliva (produced by chewing paraffin) mixed with powdered enamel and some sugar, is kept at a temperature of 37° C. for 4 hours. Caries susceptibility (i.e., the production of acid by acidogenic bacteria) is indicated by an increased calcium concentration of the saliva allegedly through solution of this mixture; caries resistance on the other hand (i.e., absence of acidogenic bacteria) is shown by a decreased calcium concentration.

The literature suggests that HA is precipitated from the saliva in vivo: Hevesy and his collaborators,[18, 19] as well as Erbacher and Wannenmacher,[20] by means of radioactive phosphorus (P^{32}) were able to demonstrate crystallization of phosphate from the saliva onto the enamel as well as into the enamel. After injection of P^{32} the enamel of erupted teeth became radioactive by the way of the saliva and not from the pulpal side. The possibility that HA is

precipitated from the saliva onto the enamel is further suggested by the observation made by Wannenmacher[21, 22] that after injection of radioactive phosphate the whole human body showed radioactivity, but only those teeth became radioactive which had not been protected from the saliva by metal caps. Furthermore Volker and Sognnaes[23] found, after injection of P^{32}, that the surface layer of enamel was more radioactive than the inner layers.

If saliva and enamel are to be regarded as a physico-chemical system consisting of a liquid and a solid phase the oversaturation state of saliva eventually could engender remineralization of enamel. Since however enamel is not a pure inorganic phase due to its content of protein, the contention that oversaturation of the saliva causes remineralization needs adequate in vivo experimentation.

SUMMARY

Unstimulated and stimulated saliva of persons resistant to dental caries was brought into contact with pure hydroxyapatite in vitro. The concentrations of calcium and phosphate were decreased in both kinds of saliva. However, in samples of stimulated saliva the decrease was greater than in that of unstimulated saliva. This phenomenon was shown to be due to the fact that both kinds of saliva represent oversaturated solutions, in which hydroxyapatite is crystallized by the addition of hydroxyapatite crystals. Unstimulated saliva is less oversaturated than stimulated saliva due to its higher dissolving capacity. This in return is induced by the lower pH and higher buffering capacity of unstimulated saliva.

The in vivo-analogy of a process of oversaturation of saliva with hydroxyapatite would point to a possible mechanism of remineralization of the enamel but requires in vivo demonstration.

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IN VITRO STUDIES ON THE PROTECTION OF PENICILLIN-SENSITIVE ORAL ACIDOGENIC BACTERIA BY YEASTS PRESENT IN HUMAN SALIVA

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THE STATUS of penicillin as a human dental caries-preventing agent is beclouded by conflicting reports and by inconsistencies between the clinical and bacteriologic findings.[1-4] Hill's observations that oral strains of lactobacilli are extremely sensitive to penicillin have been amply confirmed by many investigators.[5] Yet, reductions in dental caries incidence in users of penicillin-containing dentifrices have not been accompanied by corresponding changes in salivary lactobacillus counts.[6, 7] The enigma was partially resolved by studies in this laboratory which showed that oral lactobacilli can develop a tolerance to penicillin when repeatedly exposed to the concentrations present in penicillin dentifrices.[8]

The human oral flora is comprised of penicillin-sensitive and penicillin-insensitive organisms. Foster and Woodruff[9] noted that penicillin is entirely inactive against yeasts, pathogenic and saprogenic fungi. Glass,[10] and Young, Resca, and Sullivan[11] reported that from 45 to 50 per cent of human saliva samples contain yeasts with *Candida albicans* predominating and Cryptococci and Saccharomyces found on occasion. The inhibiting effect of penicillin on the growth of some bacteria has been shown to be relieved by a factor contained in yeast extract.[12] Can penicillin-insensitive oral yeasts protect penicillin-sensitive oral acidogenic bacteria against inhibitory concentrations of penicillin? Is the protection complete or partial? The present investigation was designed to evaluate this hypothesis.

MATERIALS AND METHODS

The penicillin sensitivity of pure cultures of previously unexposed oral strains of *Lactobacillus acidophilus, Streptococcus salivarius, Staphylococcus albus, Candida albicans* and *Saccharomyces cerevisiae* was determined in 2 groups of 5 series of duplicate tubes. One group contained 10 ml. per tube of glucose-enriched thioglycollate broth; the other, 10 ml. per tube of double strength Landy and Dicken broth.[13] Penicillin-G-potassium was added to each series in amounts ranging from 1 μg through 25 mg. per tube. Each series was seeded with 1 drop per tube of a 24-hour thioglycollate broth culture of a test organism. The tubes were examined turbidimetrically after incubating

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at 37° C. for 72 hours. The lowest concentration of penicillin which completely prevented the growth of a test organism was termed the minimum inhibitory level.

TABLE I

EFFECT OF ADDED PENICILLIN-G-POTASSIUM ON ACID PRODUCTION IN A SYNTHETIC MEDIUM* BY MIXED CULTURES OF ORAL ACIDOGENIC BACTERIA AND ORAL YEASTS

ORGANISMS†	ACID FORMED IN 72 HOURS (ML. 0.1 N ACID/10 ML. BROTH)					
	NO PGP‡	0.5 MG. PGP	1.0 MG. PGP	5.0 MG. PGP	10.0 MG. PGP	25.0 MG. PGP
Candida albicans	0.6	0.5	0.5	0.3	0.3	0.3
+ Group I	16.4	1.0	0.3	0.4	0.4	0.3
+ Group II	16.0	0.7	0.3	0.3	0.4	0.4
+ Group III	14.8	0.7	0.4	0.4	0.5	0.4
+ Group IV	11.3	0.7	0.3	0.2	0.3	0.4
Saccharomyces cerevisiae	1.2	1.1	0.3	0.3	0.2	0.2
+ Group I	16.8	0.8	0.0	0.0	0.0	0.0
+ Group II	15.7	0.9	0.0	0.0	0.0	0.0
+ Group III	13.0	1.0	0.0	0.0	0.0	0.0
+ Group IV	14.2	1.1	0.0	0.0	0.0	0.0

*Double strength Landy and Dicken broth.
†Group I—*Lactobacillus acidophilus.*
Group II—*Lactobacillus acidophilus, Streptococcus salivarius, Staphylococcus albus.*
Group III—*Lactobacillus acidophilus, Staphylococcus albus.*
Group IV—*Lactobacillus acidophilus, Streptococcus salivarius.*
‡PGP=Penicillin-G-potassium.

To determine whether organisms susceptible to penicillin in pure culture can withstand levels of this antibiotic in excess of the minimum inhibitory concentrations when grown in mixed culture, the test bacteria were grouped with either *Candida albicans* or *Saccharomyces cerevisiae*. The experimental procedure was essentially the same as described above for determining the sensitivity of pure cultures of each organism. The composition of the mixed cultures is shown in Table I. Penicillin-G-potassium was added to each series in the amounts shown in Figs. 1 and 2. The tubes were incubated at 37° C. for 72 hours at the end of which time the organisms were identified from Gram's stains of the mixed culture. Acid production was measured in the penicillin-containing tubes of Landy and Dicken broth by titration with 0.1 N NaOH to the pH of uninoculated control tubes. The quantity of acid formed by the same combinations of organisms in penicillin-free Landy and Dicken broth served as a standard of reference. A Beckman pH meter was used for this purpose.

Assays were performed to establish whether the potency of penicillin was affected by the experimental procedure and whether penicillin was inactivated by any of the test organisms. The determinations were made by the method of Bond and Davies[14] using *Staphylococcus aureus* NRRL strain B-313 (ATCC #6538P) as the assay organism. Both penicillin-containing uninoculated thioglycollate broth and penicillin-containing thioglycollate broth inoculated with the 3 test bacteria and with either *Candida albicans* or *Saccharomyces cerevisiae* were measured for penicillin content following incubation at 37° C. for 72 hours. Prior to inoculation with the assay organism, each broth was filtered through a bacteriologic fritted glass filter. The penicillin content was determined from a standard curve developed for each assay.

OBSERVATIONS

As shown in Figs. 1 and 2, the minimum concentrations of penicillin-G-potassium which completely inhibited growth of the test organisms in pure culture were: *Lactobacillus acidophilus,* 7.5 μg in Landy and Dicken broth and 10 μg in thioglycollate broth; *Staphylococcus albus,* 2.5 μg in each

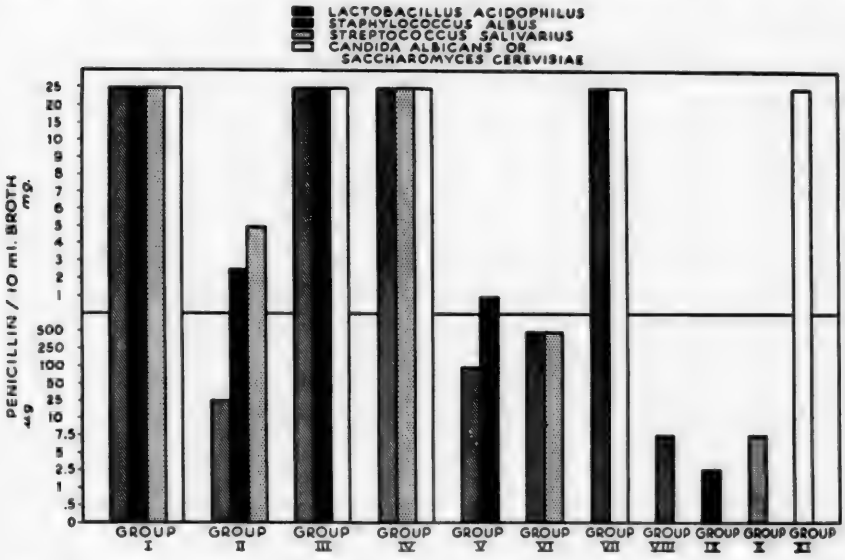

Fig. 1.—Growth of mixed oral acidogens in penicillin-containing synthetic media (double strength—Landy and Dicken broth).

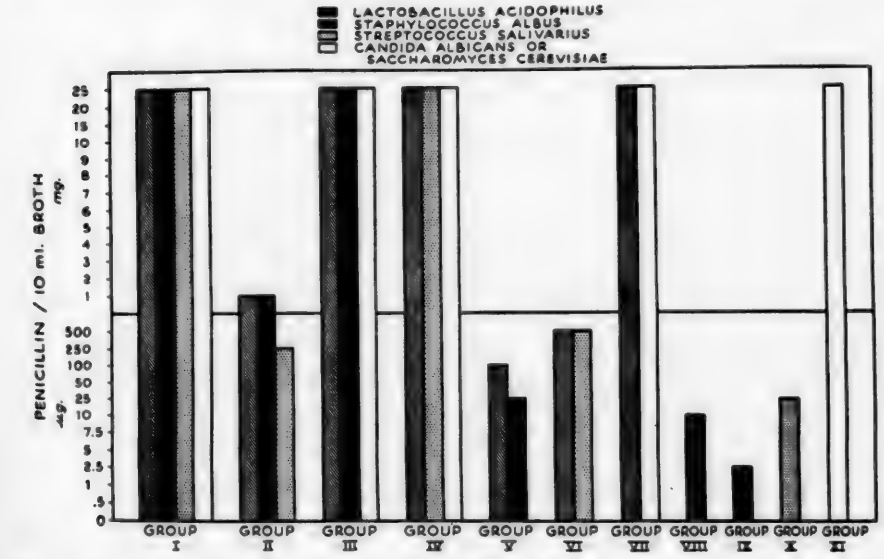

Fig. 2.—Growth of mixed oral acidogens in penicillin-containing synthetic media (thioglycollate broth).

medium; *Streptococcus salivarius,* 7.5 μg in Landy and Dicken broth and 25 μg in thioglycollate broth. The strains of *Candida albicans* and *Saccharomyces cerevisiae* were not inhibited in either broth by quantities up to and including 25 mg. of penicillin-G-potassium per tube.

When grown in mixed culture with either or both the test strains of *Staphylococcus albus* and *Streptococcus salivarius,* the minimum amount of penicillin required to completely inhibit *Lactobacillus acidophilus* exceeded that which produced an identical effect in pure culture. Tolerance to penicillin of the previously unexposed strain of *Lactobacillus acidophilus* increased 3 to 13 and 10 to 100-fold in Landy and Dicken and glucose-enriched thioglycollate broth, respectively, in the presence of one or both of these organisms. Mixtures of the 3 bacteria were more resistant to penicillin in each medium than were combinations of *Lactobacillus acidophilus* and either *Staphylococcus albus* or *Streptococcus salivarius.* Regardless of the medium and of the presence or absence of *Staphylococcus albus* and/or *Streptococcus salivarius,* the addition of *Candida albicans* or *Saccharomyces cerevisiae* to the mixed cultures enabled *Lactobacillus acidophilus* to withstand penicillin-G-potassium in quantities up to and including 25 mg. per tube. The maximum experimental level of penicillin (25 mg. per tube) also failed to inhibit completely the growth of penicillin-sensitive *Staphylococcus albus* or *Streptococcus salivarius* in the presence of either of the test strains of yeast (Figs. 1 and 2).

Although the growth of the test bacteria was not completely arrested by comparatively huge amounts of penicillin in the presence of *Candida albicans* or *Saccharomyces cerevisiae,* acid production by these organisms was reduced to negligible quantities or completely prevented by penicillin in amounts far below those which failed to exert a complete bacteriocidal effect. As shown in Table I, the addition of 500 μg of penicillin-G-potassium to 10 ml. of Landy and Dicken medium materially reduced acid production by the mixed cultures irrespective of the component organisms. Neither strain of yeast was an appreciable acid producer either in the presence or absence of penicillin.

The effect of the experimental procedure and of the test organisms on the quantitative recovery of penicillin-G-potassium added to glucose-enriched thioglycollate broth is presented in Figs. 3 and 4. Passage of the penicillin-containing broth through a bacteriologic fritted glass filter did not alter the penicillin content of the broth so treated. The spontaneous breakdown of penicillin-G-potassium in broth incubated at 37° C. for 24, 48, and 72 hours was negligible. No noteworthy reduction in penicillin concentration occurred in broth seeded with pure cultures of *Candida albicans* and *Saccharomyces cerevisiae* or with a mixed culture of *Candida albicans, Lactobacillus acidophilus, Streptococcus salivarius* and *Staphylococcus albus.*

DISCUSSION

While the precise mode of action of penicillin is as yet not known, available evidence suggests that it acts by inhibiting an early stage of nucleic acid synthesis. Ribose nucleic acid is the primary nucleic acid affected.[15] The nucleic acid content of bacteria surpasses that of nearly all types of cells. Thus

bacteria are extremely vulnerable to an agent which interferes with their synthesis of these compounds. Since yeasts are a prime source of ribose nucleic

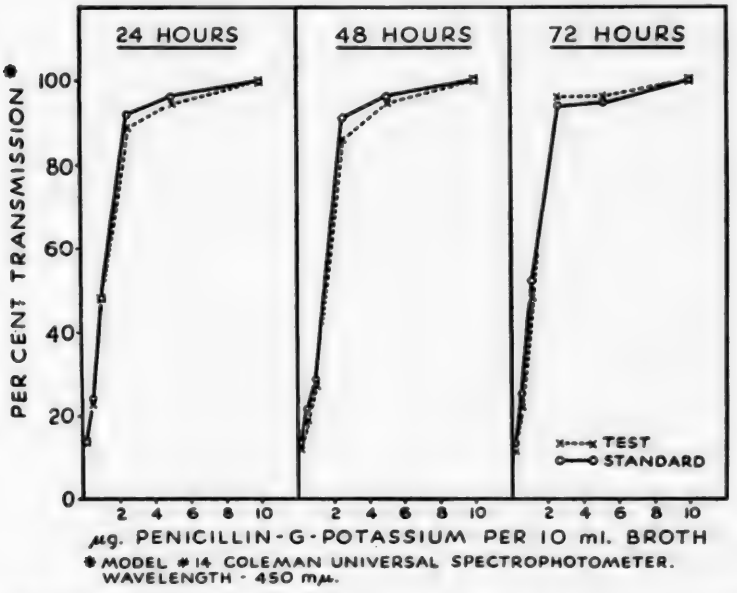

Fig. 3.—Effect of incubation at 37° C. on quantitative recovery of penicillin-G-potassium added to glucose-enriched thioglycollate broth.

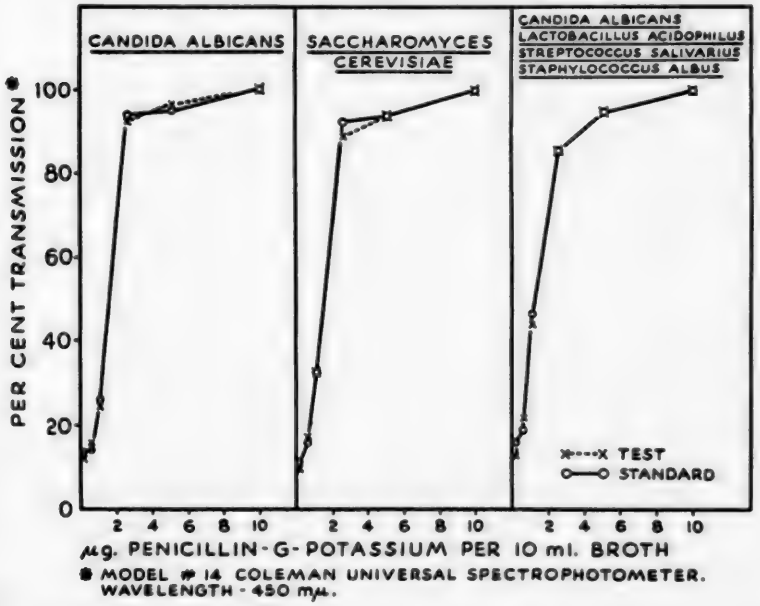

Fig. 4.—Effect of test organisms on quantitative recovery of penicillin-G-potassium added to glucose-enriched thioglycollate broth.

acid and are not sensitive to penicillin, it would appear that the presence of these organisms in mixed culture provided the susceptible bacteria with nucleic acid precursors or constituents which thereby prevented complete extinction.

Growth and acid production by oral acidogenic bacteria, though related, are not necessarily synonymous. Different strains vary in their ability to form acid from the same substrate although their growth curves are often identical. For any given strain, acid production is maximal only under optimal conditions. In the presence of metabolic deterrents such as penicillin this characteristic is subject to mutational change. This factor may account for the noteworthy reduction in acid formation by the mixed cultures of oral acidogens grown in the presence of exceedingly high levels of the antibiotic.

SUMMARY AND CONCLUSIONS

When grown in mixed culture, penicillin-resistant oral strains of *Candida albicans* or *Saccharomyces cerevisiae* enabled sensitive oral strains of *Lactobacillus acidophilus, Streptococcus salivarius* and *Staphylococcus albus* to withstand concentrations of penicillin bacteriocidal in pure culture.

Despite their viability in the yeast-containing mixed cultures, acid production by the test strains of acidogenic bacteria was virtually eliminated by penicillin in concentrations exceeding the minimum inhibitory level for pure cultures of each of the constituent bacteria.

Protection afforded penicillin-sensitive oral acidogenic bacteria by resistant oral yeasts should be considered in the evaluation of salivary lactobacillus counts in studies of the dental caries-preventing potential of penicillin.

We are indebted to Ellie Jo Gilley and Jenny MacGregor for technical assistance.

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STUDIES OF THE COMPOSITION OF TEETH

III. The Amino Acid Composition of Human Dentinal Protein

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THERE has been much interest in the nature of the organic matrices of tooth tissues, for they are thought to be intimately involved in dental caries. However, only recently has the chemical composition of these matrices been studied in any detail. Human dentin averages 21 to 22 per cent organic matrix, of which 1 to 2 per cent is nonprotein, consisting mostly of citric acid, mucoid, lipid, and cholesterol.[1-7] Therefore, quantitative determination of the amino acids of dentin would account for much of its organic matrix and would indicate its chemical nature.

Qualitative analyses which have been made of the amino acids of dentinal protein are not entirely in agreement. Atkinson and Matthews[8] found 18 amino acids, including tryptophane but not cystine, in dentin by qualitative paper chromatography. Losee, Neidig, and Hess[9] were able to account for 13 amino acids in dentin by quantitative colorimetric and microbiologic methods. More recently, however, Hess, Lee, and Niedig,[10] utilizing similar methods, accounted for 20 amino acids in dentin, including cystine but not tryptophane. All such analyses have been made of the residue of acid-decalcified dentin, whose amino acid composition is likely to be affected by such decalcifying processes. Since powerful chelating agents have become available, it is now possible to decalcify tooth tissues at or near neutrality, with less chance of influencing their amino acid composition. In the following report a quantitative determination has been made of the amino acid content of dentin decalcified by a chelating agent near neutrality and of a water soluble extract of powdered dentin.

METHODS

Dentin for analysis was obtained from recently extracted, caries-free, permanent human teeth collected at several military establishments from men over 18 years of age. Immediately after extraction, the teeth were rinsed in water, dried, and placed in a desiccator containing anhydrous $CaSO_4$ for no longer than 24 hours, after which they were frozen at –5° C. Before separating dentin from enamel and cementum, the teeth were thawed, and scrubbed thoroughly in soap and water with a brush, after which most of the cementum was removed by steel burs revolving at slow speed.

The research reported is an abridgment of a portion of a dissertation submitted by Gino Battistone in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Georgetown University.

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The teeth were then prepared for gravitational flotation separation of dentin from other tissues by grinding in a Wiley Mill to a suitable particle size which passed a 60-mesh screen but did not pass a 100-mesh screen. Separation of large quantities of tooth tissues was accomplished in a flotation apparatus devised from 2 interconnecting separatory funnels (Fig. 1). This

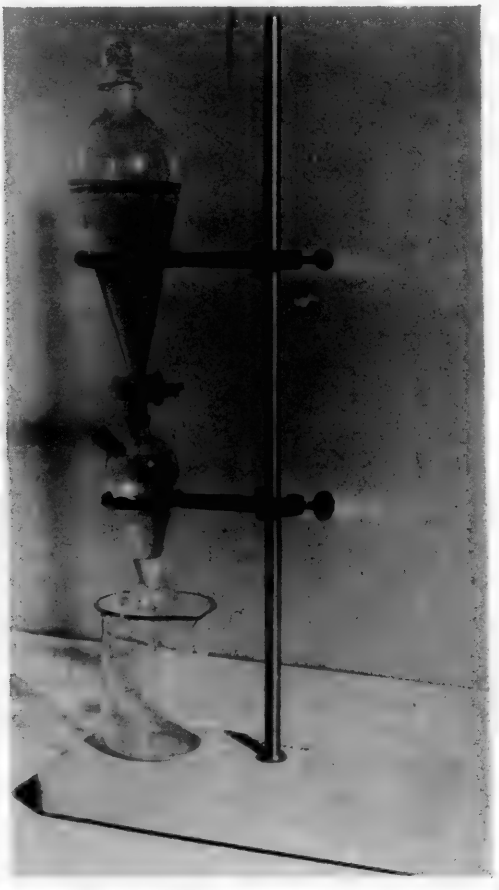

Fig. 1.—Flotation apparatus consisting of 2 interconnecting separatory funnels filled with a mixture of S-tetra bromoethane and acetone of the desired specific gravity.

apparatus was filled with a mixture of 87.8 per cent S-tetra bromoethane and 12.2 per cent acetone (specific gravity, 2.70). When the powdered teeth (+60, –100 mesh particles) were introduced into the top of the fluid, enamel was continuously separated from dentin by the force of gravity and could be removed from the bottom separatory funnel. Subsequent purification of dentin was made in a similar fluid whose specific gravity was adjusted to 2.42, resulting finally in dentin of 99.9 per cent purity as determined by microscopic particle count. This method of the separation of large quantities of

tooth tissues does not require centrifugation as have other flotation procedures (Manly and Hodge[11]), and allows ease and efficiency in separating tooth tissues.

The separated and dried dentin was prepared for analysis: (1) by decalcifying in 15 per cent ethylenediamine tetraacetic acid (EDTA)* at room temperature, pH 7.0 to 7.5, for 4 days; or (2) by extracting in boiling water for 24 hours. The decalcifying apparatus consisted of Visking dialyzing membrane spread over the end of a large glass tube. The tube containing the dentin sample and 10 to 15 ml. of decalcifying solution was attached to a stirring motor and placed in a container of the decalcifying solution so that the liquid levels inside and outside the dialyzing tube were equal. The tube was rotated at intervals of 15 seconds to hasten the rate of decalcification. The criterion for complete decalcification was the titration of an EDTA excess with standardized $CaCl_2$ until a constant EDTA value was achieved. After decalcification, the residual dentin was washed thoroughly in distilled water and dried to a constant weight at 100° C.

Dentin for analysis was prepared also by an alternate method of refluxing purified powdered dentin in distilled water for 24 hours. The solution was filtered and concentrated to ¼ volume under reduced pressure, and the protein precipitated by the addition of 10 volumes of absolute alcohol. Complete precipitation occurred in 2 to 4 hours at 5° C., after which it was centrifuged and washed 4 or 5 times with cold 50 per cent alcohol. The supernatant was decanted and the precipitate was dried to a constant weight at 100° C. The precipitate was yellowish and resembled gelatin. It was readily soluble in warm water but would not gel at 5° C.

The 2 types of residual dentin were each hydrolyzed for 16 hours at 115° C. in sealed bombs containing 50 times as much 20 per cent HCl as dentinal protein. Cystine and methionine were not detectable while histidine and tyrosine were barely detectable in such hydrolysates. To preserve these amino acids, dentin residues were hydrolyzed for only 6 hours in 20 per cent HCl at 115° C. All acid hydrolysates were oxidized for 4 hours at room temperature with sufficient 30 per cent H_2O_2 to equal to ⅕ the volume of the hydrolysate, resulting in the production of cysteic acid and methionine sulphone. Since tyrosine is affected by oxidation, its concentration was determined from aliquots obtained before oxidations of the hydrolysates. Dentinal residues were also hydrolyzed at 115° C. for 6 hours in 5N NaOH to preserve any tryptophane which might be present.

The amino acids of the various hydrolysates were determined by the quantitative, two-dimensional chromatographic method of Wellington.[12] Modifications were made in the method by using descending rather than ascending chromatographs, and by the lengthening of solvent runs so that better separation could be obtained for some amino acids. The first dimensional run in an alcohol water-ammonia mixture was carried to 35 cm. past the point of application of the hydrolysate. The second dimensional run in phenol

*EDTA was supplied by the Bersworth Chemical Co. of Framingham, Mass., under the trade name Versene.

was carried to 30 cm. past the point of application of the hydrolysate. Phenylalanine, leucine, and isoleucine were not separated by this procedure but they were separated by one-dimensional chromatograms employing a mixture of equal volumes of normal butanol and benzyl alcohol as solvent in an atmosphere of HCN.[13] The amino acid residues on the paper chromatograms were treated with 2 per cent ninhydrin in absolute alcohol and eluted with 50 per cent aqueous propanol. The eluted color was measured in a 1 cm. cell in a Beckman spectrophotometer (Model DU) with tungsten light source, at 570 mμ for the α-amino acids while proline and hydroxyproline were measured at 350 mμ. Total nitrogen in the residues was determined by the micro-Kjeldahl method of Kemmerer and Hallet.[14]

TABLE I
AMINO ACIDS IN HUMAN DENTINAL PROTEIN
(EXPRESSED AS GRAMS PER CENT OF RESIDUE, LESS WATER OF HYDROLYSIS)

AMINO ACIDS	AMINO ACID RESIDUES IN EDTA-DECALCIFIED DENTIN	AMINO ACID RESIDUES IN SOLUBLE DENTIN PROTEIN
Cystine	0.10 ± .03	0.12 ± .03
Arginine	8.3 ± .62	8.3 ± .48
Histidine	0.63 ± .10	0.60 ± .08
Lysine	3.0 ± .21	2.9 ± .17
Aspartic	6.6 ± .57	6.4 ± .61
Glutamic	10.5 ± .27	10.4 ± .22
Glycine	19.1 ± 1.19	18.9 ± 1.24
Alanine	8.5 ± .43	8.2 ± .49
Serine	2.8 ± .28	2.9 ± .25
Threonine	2.1 ± .20	2.0 ± .19
Valine	2.6 ± .25	2.5 ± .23
PLI*	8.2 ± .51	7.7 ± .56
Methionine	0.64 ± .06	0.68 ± .08
Tyrosine	1.0 ± .12	0.90 ± .13
Proline	10.0 ± 1.20	9.5 ± .89
Hydroxyproline	14.0 ± .81	13.8 ± .73
Hydroxylysine	1.1 ± .08	1.1 ± .08
TOTAL RESIDUES	99.1	96.9
ASH	1.6	1.1
TOTALS	100.7	98.0
N Calculated	17.77	17.21
N Found	18.10	17.74

*Phenylalanine, leucine, and isoleucine.

RESULTS AND DISCUSSION

The amino acid composition of the dried human dentinal residues is presented in Table I as grams per cent, calculated to exclude water acquired during hydrolysis. The data are uncorrected for ash. Four complete analyses were done in duplicate for each type of dentinal residue and the results are presented as means with their standard deviation. Total nitrogen found in each dentinal residue is also listed in Table I, together with the nitrogen content calculated on the basis of the total amino acids found in dentinal protein. The calculated nitrogen accounts for 98.18 per cent of the total nitrogen in EDTA decalcified dentin and for 97.01 per cent of the total nitrogen in the water extract of powdered dentin. It can be seen that the amino acid composition of the water extract of dentin and that of the EDTA decalcified

dentin is almost identical. No nitrogen could be detected in the supernatant after the water extract of dentin was precipitated with tannic acid. This indicates that the amino acids were not lost during extraction and that the water extract of dentin represents essentially the true amino acid composition of intact dentinal protein. Since the amino acid content of EDTA decalcified dentin and the water extract of dentin are similar, it is apparent also that EDTA does not greatly influence the amino acid composition of dentin during decalcification. Since the amino acid composition of human dentinal protein resembles that of various collagens (see the analyses of Neuman[15]), it seems logical to conclude that the organic matrix of dentin is essentially collagenous.

SUMMARY

The amino acid composition of human dentinal protein residues, obtained by decalcification with ethylenediamine tetraacetic acid (EDTA) and by refluxing in distilled water, was determined by quantitative, two-dimensional paper chromatography. Twenty amino acids were found, and the composition of the 2 residues was quite similar. The amino acids of EDTA decalcified dentin and water extracted dentin accounted for 98.18 per cent and 97.01 per cent of the respective total nitrogen. The amino acid composition of dentin is quite similar to that of collagen.

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STUDIES OF THE COMPOSITION OF TEETH

IV. The Amino Acid Composition of Human Enamel Protein

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HUMAN enamel contains less than 1 per cent organic matrix, most of which seems to be protein. Partially on the basis of its origin from epithelial tissue and partially on the basis of its physical and chemical properties, enamel organic matrix is considered to resemble most closely keratins and more specifically the eukeratins.[1-7] Eukeratins have been characterized by their histidine:lysine:arginine ratio of 1:4:12.[8] Therefore, to establish the chemical nature of the enamel organic matrix as a keratin, it is necessary to determine its amino acid composition.

Several widely varying reports are found in the literature on the amino acid composition of human enamel protein. Hess, Lee, and Neidig,[6] using colorimetric and microbiologic methods, found that the histidine:lysine:arginine ratio of the protein residue of acid-decalcified enamel was 1:4:5. Stack,[7] after treating the isolated protein residue of acid-decalcified enamel to remove "collagenous impurities," found that its amino acid composition differed from that reported by Hess and associates[6] and that its histidine:lysine:arginine ratio was essentially 1:4:12, typical of eukeratins. Both Hess and associates,[6] and Stack[7] found proline and hydroxyproline in enamel protein. Block, Horwitt, and Bolling[9] found that human enamel decalcified with 5 per cent nitric acid and treated with proteolytic enzymes decreased one-third and had a histidine:lysine:arginine ratio of 1:3:10. Since we have found that decalcification with ethylenediamine tetraacetic acid (EDTA*) has minimal effect upon the amino acid composition of human dentinal protein, EDTA was utilized to prepare enamel protein for analysis of its amino acid content by quantitative, two-dimensional paper chromatography. The results of this investigation will be reported.

METHODS

Enamel for analysis was obtained from freshly extracted, caries-free, permanent teeth, collected according to previously described procedures.[10] Enamel was separated from dentin by chipping, after which any remaining particles of dentin were ground away with steel burs. The purity of the enamel chunks was carefully determined with a dissecting microscope. As an added check, representative samples of the enamel were ground in Wiley

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*EDTA was supplied by the Bersworth Chemical Co. of Framingham, Mass., under the trade name Versene.

Mill to +60, −100 mesh particle size and the purity was found to be 99.9 per cent according to the microscopic particle count method of Manly and Hodge.[11]

Purified enamel was decalcified with 15 per cent EDTA at pH 7.0 to 7.5 for 5 days by the method previously utilized for decalcification of dentin.[10] Approximately 550 Gm. of the disodium salt of EDTA was used for chelating each mole of calcium. This represents a 50 Gm. excess of the amount of EDTA required for complete chelation. The decalcified enamel residue was washed thoroughly with distilled water and dried to a constant weight at 100° C. The criterion for complete decalcification was the titration of the EDTA excess with standardized $CaCl_2$ until a constant EDTA value was achieved.

Enamel residues were hydrolyzed at 125° C. in sealed bombs containing 50 times as much 20 per cent HCl as residue. After hydrolyzing for 10 hours, the concentration of cystine, methionine, and tyrosine decreased, and it was necessary to limit hydrolysis of the enamel residues to 8 hours for optimum recovery of these amino acids. Decalcified enamel was also hydrolyzed at 115° C. for 6 hours in 5N NaOH for optimal recovery of tryptophane.

Acid hydrolysates of enamel residue were oxidized for 4 hours at room temperature with sufficient 30 per cent H_2O_2 to equal ⅕ the total volume of the hydrolysate, resulting in the formation of cysteic acid and methionine sulphone. Since tyrosine is affected by oxidation, aliquots of the hydrolyzed enamel residue were removed for analysis of this amino acid prior to oxidation.

The amino acids of enamel were determined by the quantitative chromatographic method of Wellington,[12] modified as previously described for the determination of the amino acids of dentin.[10] Total nitrogen was determined by the ultramicro method of Seligson and Seligson.[13]

RESULTS AND DISCUSSION

The amino acid composition of human enamel protein is presented in Table I as grams per cent, calculated to exclude water acquired during hydrolysis. The data are uncorrected for ash. The results are presented as means, with their standard deviation, of 4 complete analyses made in duplicate. The nitrogen content of the decalcified enamel residue is presented as that determined by analysis and as that calculated from its amino acid composition. The amino acids of enamel account for 99.21 per cent of the total nitrogen found in EDTA-decalcified enamel.

Nineteen amino acids were determined in the decalcified enamel residue. Tryptophane was found in alkaline hydrolysates but its exact concentration could not be established. The histidine:lysine:arginine ratio of enamel protein is 1:4:12, which is the ratio of eukeratins. The presence of proline and hydroxyproline in enamel protein would indicate, however, that it is not a true keratin. The low-cystine and high-glycine content of enamel protein is also not typical of eukeratins. While enamel protein is of ectodermal origin and would, therefore, be expected to be a keratin, its composition does not conform to the usual concepts of true keratins even when it is "purified" by the removal of collagenous impurities, as was done by Stack.[7] Further speculation on the true nature of enamel protein would be of little value without further analytic data.

TABLE I
AMINO ACID RESIDUES IN ENAMEL PROTEIN
(EXPRESSED AS GRAMS PER CENT, LESS WATER OF HYDROLYSIS)

Cystine	0.60	± .03
Arginine	7.0	± .56
Histidine	0.56	± .08
Lysine	2.5	± .20
Aspartic	5.2	± .52
Glutamic	10.1	± .24
Glycine	19.7	± 1.31
Alanine	9.6	± .59
Serine	6.4	± .54
Threonine	4.3	± .35
Valine	3.4	± .23
PLI*	13.0	± .73
Methionine	1.0	± .08
Tyrosine	1.1	± .05
Proline	4.0	± .68
Hydroxyproline	5.0	± .50
Tryptophane	0.10†	
TOTAL RESIDUES	93.3	
ASH	5.8	
TOTAL RECOVERY	99.1	
Nitrogen found	16.48	
Nitrogen calculated	16.61	

*Phenylalanine, leucine, and isoleucine.
†Probable value for tryptophane.

SUMMARY

The concentration of 19 amino acids in the protein residue of ethylenediamine tetraacetic acid (EDTA) decalcified human enamel was determined by quantitative two-dimensional paper chromatography. The amino acids accounted for 99.21 per cent of the total nitrogen of enamel. The histidine:lysine:arginine ratio of enamel is 1:4:12, which is typical of eukeratins. Since enamel protein contains proline and hydroxyproline, and much glycine and little cystine, its composition is not typical of true keratins.

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STUDIES OF THE COMPOSITION OF TEETH

V. Variations in the Amino Acid Composition of Dentin and Enamel

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IN PREVIOUS articles[1, 2] we have described our methods of collecting, separating, and decalcifying tooth tissues, and have reported the amino acid composition of human dentin and enamel protein isolated at neutrality. All previous analyses of the amino acid composition of tooth tissues have been made on acid-decalcified residues of enamel and dentin.[3-5] Acid decalcification of tooth tissues is likely to cause partial hydrolysis and loss of some protein components and hence to influence the amino acid composition of enamel and dentin. To establish the amino acid composition of intact tooth tissues more definitively, it is necessary, therefore, to define the influence of various procedures used in preparing the protein residue which is finally analyzed. Such factors as the time intervening between extraction of teeth and preparation of their tissues for analysis could influence their amino acid composition. Likewise, the manner in which the teeth are preserved after extraction could also have a similar influence. Other factors which could influence the tooth tissues are their method of separation and purification and the manner of their subsequent decalcification. Some of the factors which may influence the amino acid composition of dentin and enamel protein during their preparation for analysis are compared and discussed.

METHODS

Dentin and enamel used in this study were obtained from freshly extracted teeth and from teeth, some of which had been preserved in formalin or other preservatives, and all stored for several months at 37° C. All teeth were collected at military establishments and represent fully developed teeth from men over 18 years of age.

The method of collecting and preparing freshly extracted and preserved teeth has been described previously.[1, 2] Tooth tissues were separated and decalcified by the methods of Battistone and Burnett.[1, 2] The tissues of powdered teeth were separated by a modified flotation technic or enamel was separated from dentin of whole teeth by chipping. Decalcification of the dental tissues was accomplished at room temperature by 4 different methods as follows: (1) Dentin was decalcified for 4 days and enamel for 5 days in EDTA* (pH

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*The sodium salt of ethylenediamine tetraacetic acid.

7.0 to 7.5). (2) Samples of both tissues were decalcified in 0.1 N HCl (pH 1.1) for 3 days. (3) Samples of both tissues were decalcified in 5 per cent lactic acid at pH 2.2 for 2 days. (4) Samples of both tissues were decalcified in Morse solution at pH 3.0 for 24 hours.

Approximately 550 Gm. of EDTA was used for chelating each mole of calcium. This represents a 50 Gm. excess of the amount of EDTA required for complete decalcification. One milliliter of hydrochloric or lactic acid and 0.5 ml. of Morse solution was used to decalcify each milligram of tooth tissue. After decalcification, the protein residues were washed thoroughly with distilled water and collected each time by centrifugation. All tissue residues were dried to a constant weight at 100° C.

Dentin and enamel protein residues were then hydrolyzed by previously described procedures[1, 2] prior to determining their amino acid content by the quantitative two-dimensional chromatographic method of Wellington[6] with the modifications previously described.[1] The nitrogen content of the tissue residues and soluble extracts was determined by the micro-Kjeldahl method of Kemmerer and Hallet[7] or by the ultramicro method of Seligson and Seligson.[8]

RESULTS

In the following series of comparisons of the amino acid composition of enamel and dentin residues prepared in different ways, the concentration of the acids are expressed as grams per cent, calculated to exclude water acquired during hydrolysis. All data are uncorrected for ash. Four complete analyses were made in duplicate of each residue and the results are presented as means, with their standard deviations.

In the first series of experiments, a comparison was made of the amino acid composition of dentin from freshly extracted and preserved teeth obtained by flotation or by chipping. The protein residue of each type of dentin was prepared by decalcifying with EDTA. The concentrations of lysine, glycine, alanine, and particularly proline, were decreased in the dentinal residue of preserved teeth as compared with that of freshly extracted teeth. The concentration of hydroxyproline, and the amino acid group, phenylalanine, leucine, and isoleucine, was greater in the residue from preserved teeth than it was in the residue from freshly extracted teeth. Also, the total amino acid residues and the nitrogen content of preserved material was lower than those of freshly obtained material. The method of tissue separation does not influence the amino acid content of dentin. The concentration of hydroxylysine was determined only in fresh-floated dentin decalcified in EDTA (Table I) and was estimated for the other dentinal residues.

In the next series of experiments (Table II) a comparison is made of the influence of 4 methods of decalcification on the amino acid content of protein residues from the dentin of freshly extracted teeth. The concentration of alanine and the amino acid group, phenylalanine, leucine, and isoleucine, was greatest in EDTA-decalcified dentin, while the concentration of lysine, serine, and threonine was decreased by this method of decalcification. Lactic acid

TABLE I

THE AMINO ACID COMPOSITION OF FRESH AND PRESERVED DENTIN, SEPARATED BY DIFFERENT METHODS AND DECALCIFIED IN EDTA*
(EXPRESSED AS GRAMS PER CENT, LESS WATER OF HYDROLYSIS)

AMINO ACIDS	FLOATED DENTIN		NONFLOATED DENTIN	
	FRESH	PRESERVED	FRESH	PRESERVED
Cystine	0.10 ± .03	0.10 ± .02	0.11 ± .02	0.10 ± .02
Arginine	8.3 ± .62	8.5 ± .59	8.2 ± .48	8.4 ± .65
Histidine	0.63 ± .10	0.54 ± .10	0.63 ± .08	0.59 ± .11
Lysine	3.0 ± .21	2.5 ± .19	3.0 ± .17	2.7 ± .18
Aspartic	6.6 ± .57	7.0 ± .54	6.5 ± .61	6.8 ± .59
Glutamic	10.5 ± .27	10.6 ± .33	10.4 ± .22	10.5 ± .31
Glycine	19.1 ± 1.19	17.4 ± .85	19.2 ± .93	17.3 ± 1.07
Alanine	8.5 ± .43	6.9 ± .44	8.4 ± .49	7.0 ± .40
Serine	2.8 ± .28	2.7 ± .28	2.8 ± .25	2.8 ± .20
Threonine	2.1 ± .20	2.2 ± .22	2.1 ± .17	2.2 ± .19
Valine	2.6 ± .25	2.5 ± .22	2.6 ± .23	2.4 ± .22
PLI**	8.2 ± .51	9.4 ± .63	7.9 ± .56	9.2 ± .62
Methionine	0.64 ± .06	0.62 ± .05	0.63 ± .08	0.60 ± .11
Tyrosine	1.0 ± .12	1.1 ± .13	1.0 ± .10	1.1 ± .13
Proline	10.0 ± 1.20	5.2 ± .70	10.4 ± .84	5.6 ± .77
Hydroxyproline	14.0 ± .81	16.2 ± .87	13.0 ± .73	15.9 ± .92
Hydroxylysine	1.1 ± .08	1.1***	1.1***	1.1***
Total Residues	99.1	94.6	98.8	94.2
Ash	1.6	2.0	1.5	2.0
% Recovery	100.7	96.6	100.3	96.2
N Calculated	17.77	16.41	17.70	16.42
N Found	18.10	17.59	18.15	17.48

*Ethylene diamine tetraacetic acid.
**Phenylalanine, leucine, and isoleucine.
***Values taken from fresh-floated dentin.

TABLE II

THE AMINO ACID COMPOSITION OF FRESH DENTIN DECALCIFIED IN VARIOUS MEDIA
(EXPRESSED AS GRAMS PER CENT, LESS WATER OF HYDROLYSIS)

AMINO ACIDS	DECALCIFYING MEDIA			
	EDTA*	HCl	MORSE	LACTIC ACID
Cystine	0.10 ± .03	0.11 ± .02	0.09 ± .02	0.12 ± .02
Arginine	8.3 ± .62	8.0 ± .41	7.2 ± .42	7.3 ± .40
Histidine	0.63 ± .10	0.63 ± .08	0.65 ± .09	0.58 ± .10
Lysine	3.0 ± .21	3.8 ± .21	3.9 ± .19	3.9 ± .19
Aspartic	6.6 ± .57	6.0 ± .49	6.0 ± .50	6.0 ± .47
Glutamic	10.5 ± .27	10.3 ± .35	10.3 ± .31	10.3 ± .31
Glycine	19.1 ± 1.19	18.6 ± 1.16	18.4 ± 1.18	18.2 ± 1.21
Alanine	8.5 ± .43	7.3 ± .57	7.0 ± .50	7.1 ± .51
Serine	2.8 ± .28	3.8 ± .30	3.5 ± .31	3.9 ± .32
Threonine	2.1 ± .20	3.0 ± .24	3.0 ± .23	3.2 ± .30
Valine	2.6 ± .25	2.5 ± .20	2.5 ± .24	2.6 ± .25
PLI**	8.2 ± .51	6.1 ± .58	6.4 ± .46	6.7 ± .56
Methionine	0.64 ± .06	0.62 ± .08	0.62 ± .07	0.66 ± .06
Tyrosine	1.0 ± .12	1.0 ± .14	0.90 ± .11	0.91 ± .13
Proline	10.0 ± 1.20	10.4 ± 1.13	10.0 ± 1.00	8.0 ± .83
Hydroxyproline	14.0 ± .81	13.8 ± .78	13.6 ± .70	15.4 ± .84
Hydroxylysine	1.1 ± .08	1.1***	1.1***	1.1***
Total Residues	99.1	97.1	95.2	95.9
Ash	1.6	1.7	1.5	1.4
Total Recovery	100.7	98.8	96.7	97.3
N Calculated	17.77	17.52	17.06	17.09
N Found	18.10	17.74	17.58	17.26

*Ethylene diamine tetraacetic acid.
**Phenylalanine, leucine, and isoleucine.
***Values taken from fresh-floated dentin.

decalcified-dentin contained less arginine and proline and more hydroxyproline than did EDTA-decalcified dentin. Decalcification with Morse solution caused a decrease in arginine concentration. Acid decalcification caused a greater decrease in the total amount of amino acids recovered than was caused by EDTA decalcification but more than 90 per cent of the amino acids were recovered regardless of the type of decalcification used in preparing the organic residues. There was also a reduction of the total nitrogen content of acid decalcified dentin in comparison with that of EDTA decalcified dentin.

In the next series of experiments a comparison was made of the influence of the 4 methods of decalcification on the amino acid composition of dentin from preserved teeth (Table III). The amino acid composition of the residues of preserved dentin prepared by several methods of decalcification differed from that of fresh dentin decalcified by the same methods. Glutamic and aspartic acid were decreased and valine was increased in the acid decalcified residue of preserved dentin in comparison to that of the EDTA decalcified residue of such dentin. Such variations were not found for fresh dentin decalcified similarly (Table II). The proline and hydroxyproline content did not differ between the dentin of preserved teeth decalcified by the different methods, while their concentration did vary between the dentin of freshly extracted teeth, decalcified in different ways. Proline was decreased and hydroxyproline increased in the residue of lactic acid decalcified dentin, in comparison to the residue of dentin decalcified in other ways.

TABLE III

THE AMINO ACID COMPOSITION OF PRESERVED DENTIN DECALCIFIED IN VARIOUS MEDIA (EXPRESSED AS GRAMS PER CENT, LESS WATER OF HYDROLYSIS)

AMINO ACIDS	DECALCIFYING MEDIA			
	EDTA*	HCl	MORSE	LACTIC ACID
Cystine	0.10 ± .02	0.13 ± .03	0.12 ± .02	0.12 ± .02
Arginine	8.5 ± .59	6.8 ± .51	6.4 ± .50	6.3 ± .44
Histidine	0.54 ± .10	0.53 ± .07	0.60 ± .09	0.57 ± .09
Lysine	2.5 ± .19	3.0 ± .20	3.1 ± .19	3.1 ± .20
Aspartic	7.0 ± .54	5.6 ± .42	5.8 ± .46	5.8 ± .44
Glutamic	10.6 ± .33	9.8 ± .22	9.8 ± .20	9.7 ± .22
Glycine	17.4 ± .85	17.6 ± 1.30	17.4 ± 1.33	17.0 ± 1.25
Alanine	6.9 ± .44	6.8 ± .47	6.6 ± .51	6.6 ± .41
Serine	2.7 ± .28	3.8 ± .22	3.9 ± .22	3.8 ± .26
Threonine	2.2 ± .22	3.5 ± .25	3.6 ± .23	3.7 ± .27
Valine	2.5 ± .22	3.0 ± .18	3.1 ± .20	3.1 ± .18
PLI**	9.4 ± .63	8.4 ± .57	8.6 ± .59	8.6 ± .59
Methionine	0.62 ± .05	0.66 ± .07	0.67 ± .08	0.65 ± .08
Tyrosine	1.1 ± .13	1.1 ± .15	1.1 ± .12	1.0 ± .16
Proline	5.2 ± .70	5.4 ± .66	5.4 ± .73	5.3 ± .53
Hydroxyproline	16.2 ± .87	16.1 ± .91	15.5 ± .81	15.3 ± .81
Hydroxylysine	1.1***	1.1***	1.1***	1.1***
Total Residues	94.6	93.2	92.8	91.9
Ash	2.0	2.2	2.1	2.4
Total Recovery	96.6	95.4	94.9	94.3
N Calculated	16.41	15.99	15.86	15.69
N found	17.59	16.64	16.35	16.20

*Ethylene diamine tetraacetic acid.
**Phenylalanine, leucine, and isoleucine.
***Probable value for tryptophane.

The decrease in alanine and proline and the increase in the amino acid group phenylalanine, leucine, and isoleucine of dentin of recently extracted teeth as compared to preserved dentin (Tables II and III), was consistent regardless of the method used to prepare or decalcify the tissue.

In the next series of experiments, a comparison was made of the amino acid composition of enamel from freshly extracted and preserved teeth obtained by flotation or by chipping. (Table IV). The protein residue of each type of enamel was prepared by decalcification with EDTA. There was no variation in the amino acid composition of the protein residue of enamel from recently extracted or preserved teeth. Enamel separated by flotation contained more ash and less glutamic and aspartic acid as well as less of the amino acid group, phenylalanine, leucine, and isoleucine than did the residue of enamel separated by chipping. Grinding chipped enamel in a Wiley mill resulted in a higher ash content of its protein residue.

TABLE IV
THE AMINO ACID COMPOSITION OF FRESH AND PRESERVED ENAMEL SEPARATED BY DIFFERENT METHODS AND DECALCIFIED IN EDTA*
(EXPRESSED AS GRAMS PER CENT, LESS WATER OF HYDROLYSIS)

AMINO ACIDS	FLOATED ENAMEL		NONFLOATED ENAMEL	
	FRESH	PRESERVED	FRESH	PRESERVED
Cystine	0.57 ± .09	0.58 ± .05	0.60 ± .03	0.59 ± .05
Arginine	6.9 ± .65	7.0 ± .60	7.0 ± .56	7.1 ± .31
Histidine	0.54 ± .10	0.59 ± .12	0.56 ± .08	0.54 ± .10
Lysine	2.5 ± .27	2.4 ± .29	2.5 ± .20	2.3 ± .19
Aspartic	4.2 ± .40	4.3 ± .45	5.2 ± .52	5.3 ± .49
Glutamic	8.9 ± .33	8.8 ± .32	10.1 ± .24	9.9 ± .12
Glycine	18.9 ± 1.46	18.6 ± 1.34	19.7 ± 1.31	19.5 ± 1.30
Alanine	9.3 ± .58	9.3 ± .64	9.6 ± .59	9.8 ± .60
Serine	6.2 ± .60	6.3 ± .31	6.4 ± .54	6.2 ± .52
Threonine	4.3 ± .33	4.4 ± .34	4.3 ± .35	4.2 ± .37
Valine	3.4 ± .29	3.4 ± .36	3.4 ± .23	3.4 ± .24
PLI**	11.2 ± .76	11.4 ± .94	13.0 ± .73	13.3 ± .81
Methionine	0.94 ± .17	1.0 ± .17	1.0 ± .08	1.0 ± .07
Tyrosine	1.0 ± .06	1.0 ± .06	1.0 ± .05	1.0 ± .10
Proline	3.9 ± .54	4.0 ± .62	4.0 ± .68	4.1 ± .75
Hydroxyproline	5.1 ± .49	5.2 ± .56	5.0 ± .50	5.1 ± .57
Tryptophane	0.10***	0.10***	0.10***	0.10***
Total Residues	87.9	88.4	93.3	93.3
Ash	8.5	8.3	5.8	5.7
Total Recovery	96.4	96.7	99.1	99.0
N Calculated	15.95	15.91	16.61	16.65
N Found	15.96	15.78	16.48	16.40

*Ethylene diamine tetraacetic acid.
**Phenylalanine, leucine, and isoleucine.
***Probable value for tryptophane.

The influence of 4 methods of decalcification on the amino acid content of enamel isolated from recently extracted teeth by chipping is compared in Table V. The protein residue of EDTA decalcified enamel contained less lysine and histidine and more alanine and the amino acid group, phenylalanine, leucine, and isoleucine than did the residues of acid decalcified enamel. There was more methionine and less glutamic acid in the protein residue of enamel decalcified with Morse solution and lactic acid than was present in the residue of enamel decalcified in other ways.

TABLE V
THE AMINO ACID COMPOSITION OF FRESH, NONFLOATED ENAMEL DECALCIFIED IN VARIOUS MEDIA
(EXPRESSED AS GRAMS PER CENT, LESS WATER OF HYDROLYSIS)

AMINO ACID	DECALCIFYING MEDIA			
	EDTA*	HCl	MORSE	LACTIC ACID
Cystine	0.60 ± .03	0.61 ± .02	0.63 ± .03	0.56 ± .02
Arginine	7.0 ± .56	6.6 ± .56	6.6 ± .54	6.5 ± .55
Histidine	0.56 ± .08	0.85 ± .11	0.85 ± .10	0.87 ± .14
Lysine	2.5 ± .20	3.4 ± .19	3.3 ± .22	4.1 ± .22
Aspartic	5.2 ± .52	4.9 ± .43	4.6 ± .43	5.0 ± .48
Glutamic	10.1 ± .24	9.6 ± .22	9.2 ± .22	8.6 ± .19
Glycine	19.7 ± 1.31	18.9 ± 1.38	18.9 ± .68	19.1 ± 1.33
Alanine	9.6 ± .59	8.2 ± .49	7.9 ± .52	7.8 ± .39
Serine	6.4 ± .54	6.1 ± .48	6.2 ± .51	5.8 ± .46
Threonine	4.3 ± .35	4.4 ± .32	4.9 ± .32	4.7 ± .32
Valine	3.4 ± .23	3.3 ± .28	3.3 ± .19	3.1 ± .43
PLI**	13.0 ± .73	12.0 ± .55	11.6 ± .64	11.5 ± .66
Methionine	1.0 ± .08	1.0 ± .09	1.2 ± .11	1.3 ± .11
Tyrosine	1.0 ± .05	1.1 ± .08	0.94 ± .09	0.91 ± .07
Proline	4.0 ± .68	4.2 ± .62	3.9 ± .63	3.9 ± .60
Hydroxyproline	5.0 ± .50	4.9 ± .63	5.1 ± .51	4.9 ± .53
Tryptophane	0.10***	0.10***	0.10***	0.10***
Total Residues	93.3	90.2	89.2	88.7
Ash	5.8	6.1	5.8	6.7
Total Recovery	99.1	96.3	95.0	95.4
N Calculated	16.61	16.16	15.94	15.78
N Found	16.48	16.07	16.15	16.02

*Ethylene diamine tetraacetic acid.
**Phenylalanine, leucine, and isoleucine.
***Probable value for tryptophane.

The influence of 4 methods of decalcification on the amino acid content of enamel isolated from recently extracted teeth separated by flotation procedures is compared in Table VI. There was less lysine and histidine and more alanine and the amino acid group phenylalanine, leucine, and isoleucine in the protein residue of EDTA decalcified enamel than there was in the protein residue of acid decalcified enamel. Again, there was more methionine and less glutamic acid in the protein residue of enamel decalcified with Morse solution or lactic acid than there was in the residue of enamel decalcified by other means. A comparison of the data in Tables V and VI indicates less aspartic and glutamic acid and the amino acid group, phenylalanine, leucine, and isoleucine in enamel isolated by chipping, regardless of the method of decalcification.

The distribution of nitrogen in the various solutions and residues of dentin during the process of decalcification was determined in the apparatus devised by Battistone and Burnett,[1] and these findings were compared with the total nitrogen of intact dentin previous to its decalcification (Table VII). This apparatus consisted of Visking dialyzing membrane spread over the end of a large tube and the other end attached to a stirring motor. The material to be decalcified, together with the decalcifying solution was placed in the tube. The covered end was placed in a container of decalcifying solution so that the liquid levels inside and outside the dialyzing tube were equal. In such an apparatus the nitrogen may remain as a part of the dentinal residue,

it may be solubilized but remain within the dialyzing membrane, or it may pass through the dialyzing membrane. The nitrogen distribution in 3 areas is expressed in Table VII as a percentage of that found in intact dentin. The nitrogen distribution is also expressed as a percentage of the total nitrogen found in the dentin residue and inside and outside the dialyzing membrane after the dentin had been completely decalcified. Since the EDTA itself contains considerable nitrogen, an accurate analysis of the soluble nitrogen derived from dentin was impractical in both the outer and inner chambers of

TABLE VI
THE AMINO ACID COMPOSITION OF FRESH, FLOATED ENAMEL DECALCIFIED IN VARIOUS MEDIA (EXPRESSED AS GRAMS PER CENT, LESS WATER OF HYDROLYSIS)

AMINO ACID	DECALCIFYING MEDIA			
	EDTA*	HCl	MORSE	LACTIC ACID
Cystine	0.57 ± .09	0.64 ± .05	0.63 ± .04	0.58 ± .02
Arginine	6.9 ± .65	6.7 ± .62	6.6 ± .62	6.6 ± .64
Histidine	0.54 ± .10	0.8 ± .15	0.80 ± .13	0.84 ± .13
Lysine	2.5 ± .27	3.3 ± .35	3.2 ± .33	3.9 ± .37
Aspartic	4.2 ± .40	3.8 ± .42	3.6 ± .41	3.9 ± .39
Glutamic	8.9 ± .33	8.5 ± .32	8.0 ± .27	7.7 ± .32
Glycine	18.9 ± 1.16	19.0 ± 1.35	18.8 ± 1.37	19.0 ± 1.23
Alanine	9.3 ± .58	8.2 ± .55	8.0 ± .53	7.8 ± .50
Serine	6.2 ± .60	6.2 ± .49	6.1 ± .51	5.9 ± .54
Threonine	4.3 ± .33	4.5 ± .33	4.9 ± .35	4.5 ± .32
Valine	3.4 ± .29	3.2 ± .27	3.4 ± .31	3.2 ± .30
PLI**	11.2 ± .76	9.8 ± 1.08	9.4 ± .83	9.5 ± .75
Methionine	0.94 ± .17	1.0 ± .14	1.2 ± .17	1.2 ± .17
Tyrosine	1.0 ± .06	1.0 ± .09	1.0 ± .07	0.92 ± .08
Proline	3.9 ± .54	4.1 ± .62	3.6 ± .59	3.4 ± .54
Hydroxyproline	5.1 ± .49	5.3 ± .63	5.3 ± .51	5.4 ± .58
Tryptophane	0.10***	0.10***	0.10***	0.10***
Total Residues	87.9	86.1	84.7	84.4
Ash	8.5	9.0	8.9	8.9
Total Recovery	96.4	95.1	93.6	93.3
N Calculated	15.95	15.73	15.47	15.40
N Found	15.96	15.54	15.20	15.61

*Ethylene diamine tetraacetic acid.
**Phenylalanine, leucine and isoleucine.
***Probable value for tryptophane.

TABLE VII
NITROGEN DISTRIBUTION IN VARIOUS DECALCIFYING SYSTEMS**

% N	EDTA*		MORSE		LACTIC		HCl	
	% TOTAL N IN DENTIN	% OF N IN SAMPLE	% TOTAL N IN DENTIN	% OF N IN SAMPLE	% TOTAL N IN DENTIN	% OF N IN SAMPLE	% TOTAL N IN DENTIN	% OF N IN SAMPLE
Intact dentin	2.89		2.96		3.01		3.05	
Inner fluid	0.21	7.26	0.58	19.59	0.52	17.27	0.49	16.06
Outer fluid	0.04***	1.38	0.22	7.56	0.21	6.98	0.15	5.00
Insol. residue	2.64	91.34	2.09	70.60	2.15	71.42	2.28	74.75
Total recovery		100.00	2.89	97.95	2.88	95.67	2.92	95.81

*Ethylene diamine tetraacetic acid.
**The fluids inside and outside the dialyzing membrane and the insoluble residue are analyzed.
***Determined by difference.

the decalcifying apparatus. The nondialyzable nitrogen in the inner chamber of the decalcifying apparatus was ascertained, however, after decalcification was complete by dialyzing against distilled water for 2 days at 4° C. to remove EDTA. The nitrogen remaining within the inner chamber was considered to be soluble but nondiffusable nitrogen. The total nitrogen of the inner fluid and the dentin residue deducted from the total nitrogen of intact dentin was considered to be diffusible nitrogen.

Most of the nitrogen of dentin remains with the residue after decalcification. However, some of the nitrogenous components of dentin are rendered soluble during decalcification with acids or at neutrality with EDTA. Approximately 70 to 75 per cent of the total nitrogen of intact dentin is recovered in the acid decalcified residue. More nitrogenous compounds were rendered soluble during decalcification with Morse solution than were lost during decalcification with lactic or hydrochloric acid. On the other hand, approximately 92 per cent of the total nitrogen of intact dentin was retained in the insoluble residue remaining after EDTA decalcification. A portion of the nitrogenous components of dentin which were rendered soluble during acid decalcification was diffusible but the significance of the small amounts of diffusible nitrogenous compounds produced during EDTA decalcification is unknown.

DISCUSSION

For an adequate and valid analysis of tooth tissues it is essential to maintain their amino acid composition intact during isolation of the protein residues. If it is not possible to do so, then it is necessary to define the influence of the various steps of preparation upon the amino acid composition of the protein residue which is finally analyzed.

One of the first factors to be considered is the manner in which the teeth are handled between the extraction and the initiation of procedures to separate their enamel, dentin, and cementum. It was found that storing preserved teeth influenced the amino acid composition of dentin but had little effect upon enamel. The most striking change was the decrease in proline in preserved dentin as compared to dentin from freshly extracted teeth but there was also less nitrogen and less total amino acids. While it may be that the organic matrix of preserved dentin degenerates, it is more likely that the lability of the contents of the dentinal tubules accounts for the loss of amino acids, since enamel was not significantly affected by storage.

Another factor which might influence the amino acid composition of the isolated protein residues of tooth tissues is the method of separating the tissues. Two methods were used. In one method, the tissues were finely ground, a procedure which is known to affect the solubility of some keratins and separated by flotation in fluids which might leach out labile components of the tissues. The other method of separation consisted of physically separating the tissues, followed by surface grinding with burs which might influence their solubility. Apparently the methods of separating tissues, including the fluids used for flotation, have little effect upon the organic matrix of dentin. On

the other hand, there was less glutamic and aspartic acid, and the amino acid group, phenylalanine, leucine, and isoleucine in the residue of enamel separated by flotation than there was in the residue of enamel separated by chipping. The ash content of the flotation residues was also greater than that of the chipped residues. While it may be that the fluids used for flotation may influence the amino acid content of enamel residue, there is no definite explanation as to increase of the ash content.

The different methods of decalcification with acids and with EDTA at neutrality seems to affect the amino acid composition of dentin and enamel in the same way. While changes in the amino acid composition of the tooth tissue were slight, regardless of the method of decalcification, only EDTA decalcified enamel had a histidine, lysine, arginine ratio of 1:4:12, which is typical of the eukeratins. It must be pointed out, however, that all methods of decalcification of dentin result in the solubilization of some nitrogenous material, which is not accounted for in the final analysis. The tooth tissues are affected more by acids than by neutral EDTA but both cause a solubilization of nitrogenous components during decalcification. There are other indications that acid decalcification affects the integrity of tooth tissues. Burnett[9] found no oral proteolytic bacteria which could produce a detectable action upon intact dentin but many such microorganisms readily lysed decalcified dentin. More importantly, he found proteolytic bacteria could more readily lyse decalcified dentin which was exposed to acids for longer periods than they could dentin exposed to acids for only the minimal period required to decalcify it. Evans and Prophet[10] and Prophet and Atkinson[11] found that bacterial collagenases did not affect intact dentin but readily disintegrated dentin decalcified in 0.5 N HCl. Konetzka[12] also showed that intact and EDTA decalcified dentin was resistant to bacterial collagenases and other proteolytic enzymes but he found that acid decalcified dentin was susceptible to such enzymes. When all the factors are taken into consideration both acid and neutral EDTA decalcification cause solubilization of nitrogenous components of dentin. These soluble nitrogenous components are not accounted for in the dentinal residue which is finally analyzed. Until this soluble material is definitely accounted for, it is not certain whether the amino acid composition of the decalcified dentinal residue represents the actual amino acid composition of intact dentin. Changes in the amino acid composition of enamel decalcified in various ways indicates that losses of enamel protein may also be occurring during decalcification.

SUMMARY

A comparison has been made of the influence of various factors upon the amino acid composition of human enamel and dentin. Dentinal protein was affected by preservation and storage of teeth while enamel protein was not changed by such treatment. Flotation procedures did not alter the amino acid composition of dentin, but changes were noted in the amino acid composition of enamel. Therefore, separation by chipping is a more satisfactory way of separating enamel. Decalcification by acids and in the neutral sodium salt

of ethylene diamine tetraacetic acid (EDTA) causes some solubilization of nitrogenous components of dentin but EDTA decalcification causes the least change in the organic matrix of this tissue. The amino acid composition of dentin cannot be considered to be definite until the nitrogenous fraction rendered soluble during decalcification is accounted for. The same may be true for enamel protein.

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THE LIPIDE CONTENT OF ENAMEL AND DENTIN

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THE rather meager literature on the lipides in teeth has been reviewed previously by Leopold, Hess, and Carter.[1] These investigators also reported that human dentin contains 0.024 per cent cholesterol. The present paper deals with the determination of cholesterol in enamel and the total lipide content of both enamel and dentin.

EXPERIMENTAL

Sample Preparation.—The dentin used was prepared in the same manner as that previously used for the isolation of cholesterol.[1] The enamel was prepared by the method employed by Losee and Hess[2] for the isolation of enamel protein.

Cholesterol in Enamel.—The method of isolation of cholesterol from dentin that was most satisfactory was used for the isolation and estimation of the cholesterol in enamel. The procedure involved extraction of the enamel with an aqueous solution containing 30 per cent KCl and 1 per cent K_2CO_3. Twenty milliliters of solution was used for each gram of enamel. After several extractions the combined extracts were treated with 0.4 Gm. of potassium acetate, 0.4 Gm. of silica gel, and 0.4 ml. of glacial acetic acid per gram of original sample. The extract was then shaken for 30 minutes, filtered with suction through a fine-sintered glass filter, and the filtrate was extracted with petroleum ether. Both the petroleum ether extract and the aqueous extract were analyzed for total cholesterol.

To determine if the treatment with silica gel adsorbed any cholesterol, a recovery experiment using pure cholesterol was run in which 2 solutions, each containing 1.76 mg. of cholesterol in 100 ml. of H_2O, were used. One solution was treated with the same amount of silica gel used in the enamel extraction procedure and the other was used as a control. Cholesterol determinations gave a value of 1.76 mg. in the control and 1.43 mg. in the silica gel-treated solution, a loss of 18 per cent. Correction for this loss incurred in the procedure was used on the determinations of the cholesterol content of the enamel. All cholesterol determinations were made by the same method previously used.[1]

The enamel employed weighed 8.89 Gm., the aqueous extract contained 4.4 mg. of cholesterol per 100 Gm. of enamel, the petroleum ether extract

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of the aqueous extract contained 2.8 mg. per 100 Gm. The enamel remaining after the extraction procedure was then further treated with petroleum ether and this extract contained 1.1 mg. per 100 Gm. of enamel. The total yield of cholesterol was 8.3 mg. per 100 Gm. of enamel, 0.008 per cent.

Total Lipides in Dentin.—Dentin was extracted in a Soxhlet extractor with absolute ethyl alcohol for 8 hours and then with anhydrous ethyl ether for an additional 10 hours. The alcohol and ether extracts were combined, filtered, and evaporated to dryness in a tared flask. The flask and contents, dried in a vacuum desiccator for 3 days, were weighed. The dry material contains the total lipides except cholesterol which is not extracted by these solvents. Based upon several determinations on samples weighing from 4 to 5 Gm., the total lipide content was 0.26 per cent. Determination of the saponification and iodine numbers gave values of 215 and 46, respectively. Phosphorus estimation indicated that 3.06 per cent of the total lipide content was phospholipide, 0.014 per cent of the dentin.

Total Lipides in Enamel.—A sample of 200 mesh human enamel weighing 5.2273 Gm. after desiccation was extracted first with petroleum ether and then with a 1:1 mixture of anhydrous acetone and ethyl alcohol for 2 days. Evaporation of the solvents yielded 31.9 mg. of lipides, equivalent to 0.60 per cent of the enamel. The material contained no cholesterol and gave saponification and iodine numbers of 161 and 20, respectively. Phosphorus determination on an aliquot of the isolated material indicated 12.5 per cent of the total lipide was phospholipide, equivalent to 0.075 per cent of the enamel.

SUMMARY

The data on both enamel and dentin are summarized in Table I. Dentin contains 0.024 per cent cholesterol and 0.36 per cent total lipide other than cholesterol. Enamel contains only 0.008 per cent cholesterol and the non-cholesterol total lipide content is 0.60 per cent. Pincus[3] has reported the isolation of a fatty substance approximating 1 per cent of the organic fraction of enamel. His data are difficult to evaluate since his interest was primarily in the protein rather than the lipide fraction but it would appear that he found, associated with the protein, less than 0.004 per cent of the enamel as lipide.

TABLE I
CHOLESTEROL AND LIPIDE CONTENT OF ENAMEL AND DENTIN

	TOTAL LIPIDE				
	PER CENT	IODINE NO.	SAPONIFICATION NO.	PHOSPHOLIPIDE PER CENT	CHOLESTEROL PER CENT
Enamel	0.60	20	161	0.075	0.008
Dentin	0.36	46	215	0.014	0.024

The fatty acids in dentin lipide, based upon their saponification and iodine numbers, resemble those found in bone lipide, 200 and 50, respectively. The fatty acids in enamel lipide are less saturated and contain longer chains than those in bone lipides.

Krasnow[4] has reported that enamel contains more phospholipide phosphorus than dentin and our results are in agreement with this. It must be noted, however, that the values given by Krasnow in his abstract are based upon 100 mg. of enamel and dentin and unless this is a typographical error and should be based upon 100 Gm., his results are one thousand times higher than ours and would account for more than the total organic content of enamel.

CONCLUSIONS

Human enamel contains 0.008 per cent cholesterol. Total lipides other than cholesterol have been found in dentin to the extent of 0.36 per cent and 0.60 per cent in enamel. The iodine and saponification numbers of these lipides have been determined. Phospholipide contents of enamel and dentin, 0.075 and 0.014 per cent, respectively, were found, based upon the phosphorus content of the total, noncholesterol lipide.

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PHOSPHATASE, PEROXIDASE, AND OXIDASE ACTIVITY OF DENTIN AND BONE

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MOST OF the studies on enzymes in teeth have applied histochemical methods to embryonic teeth. Little work has been done on the enzyme activity in adult, fully calcified teeth using biochemical methods. MacKenzie[1] made an aqueous extract of human teeth and reported that phosphatase was found only in low concentrations in adult teeth and in somewhat larger quantities in deciduous teeth. The absolute units of enzyme activity found were not specified. Provissionate[2] also found a small amount of phosphatase in adult human teeth but Berliner[3] was unable to confirm this observation. Recently Pincus[4] reported the presence of a "dehydrogenase" and an "oxidase" in human tooth pulp and dentin. The present paper deals with the application of biochemical methods to the isolation and determination of phosphatase and respiratory enzymes in teeth and bone.

EXPERIMENTAL

Preparation of Dentin and Bone.—The coronal dentin was prepared by the method of Losee, Neidig, and Hess[5] from freshly extracted noncarious human molars. Since Pincus[4] used root dentin in his study of the respiratory enzymes this practice was repeated in our work on these enzymes. The cementum was removed from the roots with a separating disk and the root pulp ground out with a dental bur. Both the coronal and root dentin were powdered to pass 60 mesh with a Wiley mill. The dentin was usually assayed for enzyme activity the same day that the tooth was extracted, otherwise it was kept frozen solid until used.

The long bones of the mature monkey and dog and also growing rabbit and rat were all prepared for enzyme studies in the following manner; after thorough cleaning the ends were cut off and discarded, the shaft was cut lengthwise with a separating disk, the marrow removed, and the cleaned strips were dried and ground to pass 60 mesh in a Wiley mill.

Determination of Phosphatase Activity.—The method of Bessey, Lowry, and Brock[6] employing p-nitrophenylphosphate (PNPP) as substrate was used.

The data reported are taken from the thesis presented by A. A. White to the Graduate School of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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The substrate was prepared by the method of Bessey and Love[7] and purified according to the procedure of Aschaffenburg.[8] A stock solution containing 0.4 per cent PNPP in 0.001 N HCl was used.

A standard curve was prepared by pipetting from 1 to 9 ml. of a 0.1 μM per ml. aqueous solution of p-nitrophenol into 9 colorimeter tubes calibrated at 10 ml. To each tube was added 1.0 ml. of 0.02N NaOH and the volume adjusted to 10 ml. with distilled water. The tubes were read in a Klett-Summerson photoelectric colorimeter with a 42 filter.

The conditions necessary for maximum activity of bone alkaline phosphatase have been determined by Bodansky.[9] For the present work a standard assay medium was adopted consisting of 0.4 per cent PNPP, 1.0 ml.; 0.01 M $MgCl_2$, 0.4 ml.; 0.05 M glycine, 0.5 ml., and 0.1 M buffer at the selected pH, water was added to bring the final volume to 4.0 ml. It was found impossible to cover the pH range desired with a single buffer system. In the range from pH 3.2 to 9.8 the acetate-barbital-glycine buffers of Michaelis[10] were used, while for the higher pH values the carbonate-barbital-glycine buffers of King and Delory[11] were substituted. The pH values were checked with a pH meter in all cases. At the end of the assay period, usually 30 or 60 minutes, 1.0 ml. of 0.2N NaOH containing 0.05 M ethylenediamine tetracetic acid (EDTA) was added, and the reaction mixture diluted to 10.0 ml. with water. The reaction mixture was transferred to a colorimeter tube and read in the colorimeter using filter 42. The amount of p-nitrophenol released can be determined from the standard curve. Activity is expressed as μM p-nitrophenol units (μM PNP units), which are the number of micromoles of p-nitrophenol released in one hour at 37° C.

Rabbit and Rat Bone Phosphatase.—One gram of the ground bone was suspended in 10 ml. of H_2O containing 0.25 ml. of toluene. Autolysis was allowed to take place at room temperature for 2 days, after which time the suspension was filtered. Fig. 1 shows the pH-phosphatase activity curve of such an extract after it had been diluted 10 fold. There is a definite optimum at pH 10 and some slight activity at pH 5 to 6. The refrigerated filtrate maintained activity for several weeks. The dissociation constant, Ks, of the enzyme-substrate complex at pH 10.0, determined by the method of Lineweaver and Burk,[12] was found to be 5.02×10^{-4} M.

Attempts to Extract a Tooth Phosphate.—Various attempts were made to extract a phosphatase from dentin and from whole teeth. A typical experiment was the addition of 0.5 Gm. of fresh dentin to 4.0 ml. of H_2O containing a trace of chloroform or toluene to prevent bacterial growth. The suspension, frequently agitated, was allowed to remain at room temperature for 5 days and then filtered. The filtrate was assayed for activity by adding a 2.0 ml. aliquot to a colorimeter tube containing 1.0 ml. of buffer and 1.0 ml. of PNPP substrate. There was no activity either at pH 10.3 or 5.65, even after prolonged incubation at 37° C.

Similar results were obtained when the dentin from unerupted children's teeth was used. In order to obviate the possible destruction of phosphatase

in the preparation of the dentin, whole unerupted children's teeth were cleaned and powdered in the Wiley mill. The extracts prepared from these powdered teeth were also inactive.

Alkaline phosphatase is quite commonly bound to an insoluble tissue residue. Bamann, Riedel, and Diederichs[13] termed this unextractable tissue phosphatase, the desmophosphatase, as contrasted with the soluble phosphatase, the lysophosphatase. Lowry, Roberts, Nixon, Wu, and Crawford[14] found that 85 per cent of rabbit brain alkaline phosphatase was not extractable. There have been several attempts to solubilize the unextractable phosphatase. Assuming that there was a bound phosphatase present in the dentin some of these techniques were applied.

Butanol has been used to disperse the lipoprotein complex in milk which binds alkaline phosphatase.[15] Accordingly 0.5 Gm. of dentin was added to 10 ml. of H_2O and 7.0 ml. of butanol. This mixture was stirred at room temperature for 16 hours and then filtered. The filtrate was made 70 per cent with respect to ethanol and placed in the refrigerator for 3 days. The resulting precipitate was dissolved in 5 ml. of H_2O and assayed for activity; none was found.

Gold and Gold[16] have shown that collagen will adsorb phosphates from solution and that it can be partially eluted with 0.6 per cent alkyl sulfate solution. Since nearly all of the protein in dentin is collagen,[17] it is possible that the phosphatase might be associated with the collagen. When 0.5 Gm. of dentin was stirred in 15 ml. of a 0.6 per cent alkyl sulfate solution at pH 7.0 for 17 hours the supernatant solution was inactive. A powerful wetting agent, Aerosol OT, also gave negative results. Similar experiments on the mature monkey and dog bones also gave negative results for the presence of either a soluble phosphatase or an elutable bound phosphatase.

Phosphatase Activity of Dentin and Bone Powders.—Having failed to extract phosphatase from dentin, attempts were made to assay the whole fresh ground dentin directly for phosphatase activity. To 3 test tubes containing 1.0 ml. of PNPP substrate and 1.0 ml. of glycine buffer pH 10.4 were added 10, 20, and 30 mg. of dentin, respectively. After incubation at 37° C. for 1 hour the contents were diluted to 10 ml. and filtered into colorimeter tubes. Assay indicated increasing phosphatase activity with increasing amounts of dentin. Repetition of the experiment using a shaking device indicated that the activity was almost linearly proportional to the amount of dentin used.

In Fig. 2 is shown the results of an experiment with 100 mg. samples of dentin in 1.0 ml. of PNPP and 1.0 ml. glycine buffer pH 10.1. The samples were incubated for increasing periods of time. The enzyme activity, corrected for reagent blanks, is essentially linear. This curve lends support to the method of incubating the dentin itself with the substrate. The dentin used was from a pool of dried dentin and since it was found to give adequate activity the use of fresh teeth was abandoned except in special instances.

Since there was evidence for a bound phosphatase associated with dentin it was considered possible that the mature monkey and dog bone, from which

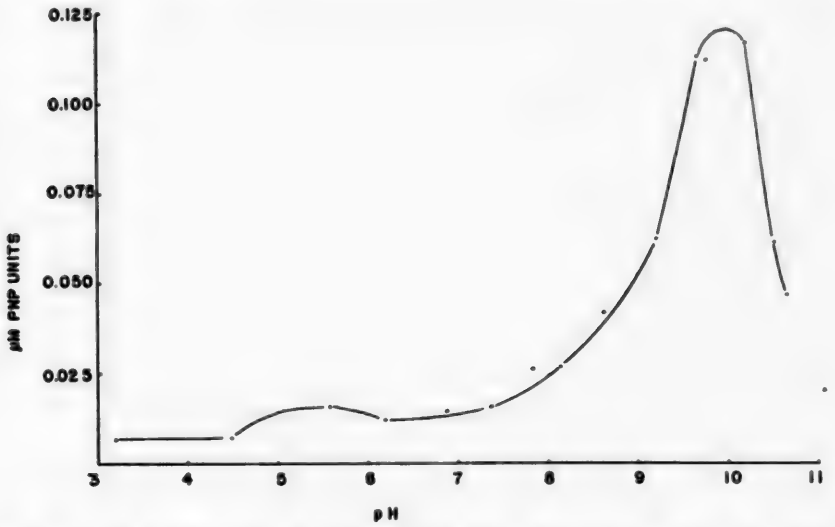

Fig. 1.—pH-Activity Curve of Soluble Rabbit Bone Phosphatase. Each point indicates the activity in μM PNP units of 0.5 ml. rabbit bone extract at that pH. The reaction mixture contained in addition, 0.00625 M glycine, 0.0125 M acetate-barbital or acetate-carbonate buffer, 0.1 per cent PNPP substrate and 0.01 M $MgCl_2$. Total volume, 4.0 ml. Incubated at 37° C. for 30 minutes. This rabbit bone extract was approximately 1 per cent, the original extract having been diluted 10 times.

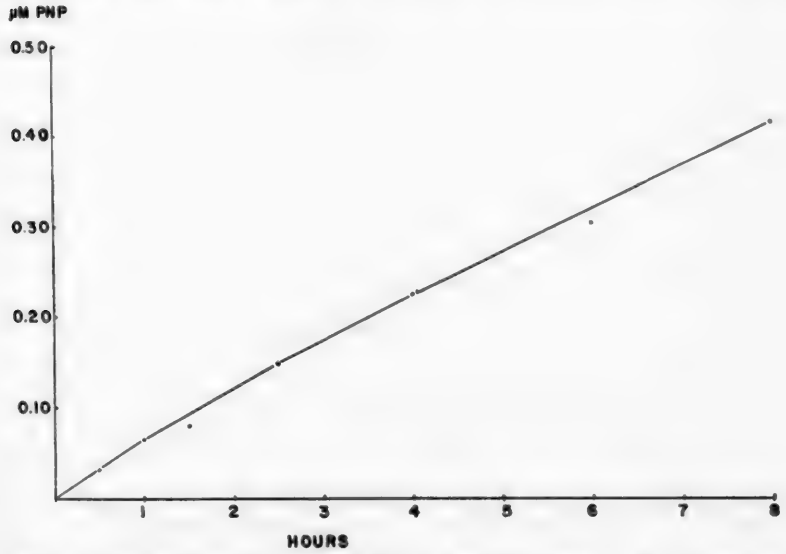

Fig. 2.—Time Phosphatase Activity Curve of Human Dentin. Each point indicates the μM PNP released by 100 mg. human dentin for that period of incubation. In addition to the dentin, each reaction vessel contained 1.0 ml. PNPP substrate and 1.0 ml. of 0.1 M glycine-NaOH buffer (containing 0.001 M $MgCl_2$), pH 10.0. Incubated at 37° C.

a soluble phosphatase could not be extracted, might also have this type of activity. Experiments, similar to those with the dentin, showed that both the monkey and the dog bone contained a bound phosphatase with twice the activity of that found in the dentin.

Comparison of pH-Phosphatase Activity Curves of Dentin and Bone.— Samples of dentin, dog, and monkey bone weighing 100 mg. were suspended in the substrate-buffer mixture at the selected pH values. The pH must be carefully adjusted after the addition of the powder using a pH meter since the calcium phosphate in the tissue becomes soluble below pH 9 and introduces an appreciable additional buffering capacity. The solutions were incubated at 37° C. for 1 hour, the reaction was then halted with EDTA and the contents filtered into colorimeter tubes after the volume had been adjusted to 10 ml. The pH-activity curve for dentin, Fig. 3, showed a broad range of activity with a maximum between pH 7 and 8, there is a much smaller peak at pH 9.5. The pH-activity curves for the dog and the monkey bones, Fig. 3, are more comparable to the soluble rabbit bone phosphatase than to the dentin.

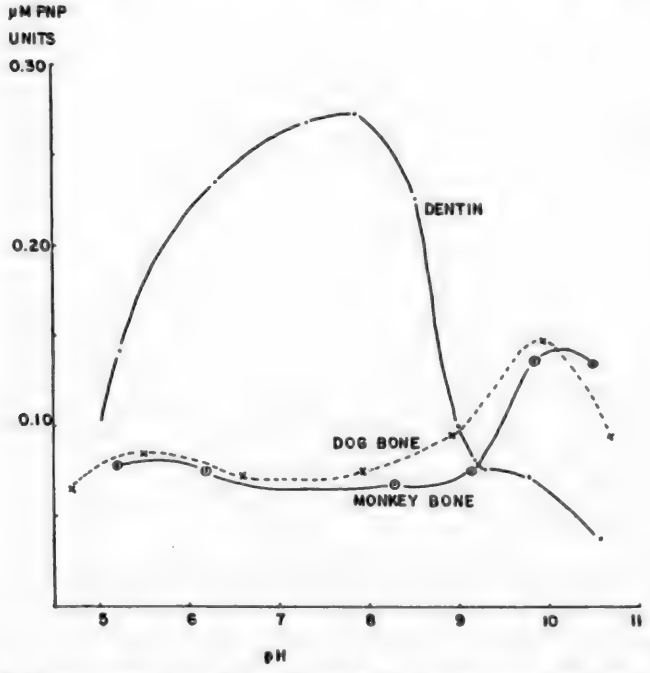

Fig. 3.—pH-Phosphatase Activity Curve of Human Dentin, Dog Bone, and Monkey Bone. Each point indicates the activity in μM PNP units of 100 mg. of the dentin or bone powder, at that pH. In addition to the dentin or bone, each reaction vessel contained 0.1 per cent PNPP, 0.00625 M glycine, 0.01 M acetate-barbital or carbonate-barbital buffer, and 0.01 M $MgCl_2$, in a total volume of 4.0 ml. Incubated 1 hour at 37° C.

To determine if the activity of the dentin in the neutral pH range was truly an enzymatic effect some of the dentin was heated in boiling water for 2 hours. It was then dried and assayed at 2 pH values, using 100 mg. samples.

The monkey and the dog bone powders were each heated and assayed in the same manner. When assayed at pH 10, Table I, the bone powders were completely inactivated by the heating while the activity of the dentin was decreased only 43 per cent. When assayed at pH 6 the bone powders were only partially inactivated while the dentin activity at pH 7.6 was decreased to approximately the same extent as at pH 10.

TABLE I. PHOSPHATASE ACTIVITY OF DENTIN AND BONE

	pH	UNHEATED μM UNITS	HEATED μM UNITS	DECREASE (PER CENT)
Dentin	10.1	0.065	0.037	43
	7.6	0.270	0.163	40
Monkey bone	10.0	0.14	0.000	100
	6.1	0.072	0.051	29
Dog bone	10.0	0.150	0.000	100
	5.9	0.080	0.032	60

It appears, therefore, that the "phosphatase" activity of both bone and dentin in the neutral range is nonenzymatic. It is of interest that EDTA, which was shown to inhibit the soluble rabbit bone phosphatase, also inhibited the nonenzymatic activity. When 0.5 ml. of 0.5 M EDTA was added to the reaction mixture and the pH was adjusted to 7.0, the activity of the dentin dropped 85 per cent and if the magnesium chloride was omitted the activity dropped 89 per cent. At pH 10.0 the EDTA completely inhibited the phosphatase activity, it might be noted that the ability of EDTA to chelate divalent metal ions varies directly with pH.[18]

In order to ensure the complete destruction of enzymes in dentin the organic matter was removed by the method of Crowell, Hodge, and Line,[19] which is said to produce little change in the inorganic portion of the dentin. The activity of the extracted dentin when assayed at pH 6.95 was almost equal to that of the unextracted dentin assayed at pH 6.87, 0.265, and 0.275 μM PNPP units, respectively.

Recently Williams and Irvine[20] have introduced a new method for preparing the inorganic matrix of bone involving extraction of the sample, in a Soxhlet extractor, with an 80 per cent aqueous solution of ethylenediamine. The method was applied to 2-gram samples of dog bone powder and dentin and the extracted, dried samples were assayed through a range of pH values from 4 to 10 (Fig. 4). The resemblance between the curves given by the extracted dentin and the bone is striking when compared to the difference in the curves obtained upon the unextracted dentin and bone (Fig. 3).

Since the inorganic matrix of both bone and dentin appeared to have phosphatase activity a synthetic hydroxyapatite was prepared according to the procedure of Lovan and Taylor.[21] Samples of the dried gel were assayed for "phosphatase" activity in the same manner as the dentin. No activity was found at pH 10.0 and 0.060 μM PNP units were found at pH 5.85. These values are much less than those obtained with the extracted dentin, 0.375

μM units at pH 5.9 and 0.035 μM units at pH 10.2. Commercially available tricalcium phosphate was also assayed and gave slight activity, 0.035 μM units at pH 6.9 and 0.025 μM units at pH 9.7.

Phosphatase Activity of Decalcified Dentin.—The possibility remained that there might be some small enzymatic phosphatase activity in dentin which was obscured by the nonenzymatic activity of the dentinal mineral. Consequently dentin was decalcified to determine whether the enzyme would remain associated with the insoluble dentinal collagen. Several decalcification experiments were performed using EDTA as the decalcifying agent. Since 30 per cent of dry dentin is calcium, twice the equivalent quantity of EDTA for 0.5 Gm. of dentin, was dissolved in 20 ml. of distilled water and brought to pH 6.5 with 10 M NaOH. The dentin and EDTA solution were stirred with a magnetic stirrer and complete decalcification was obtained within 8 to 12 hours.

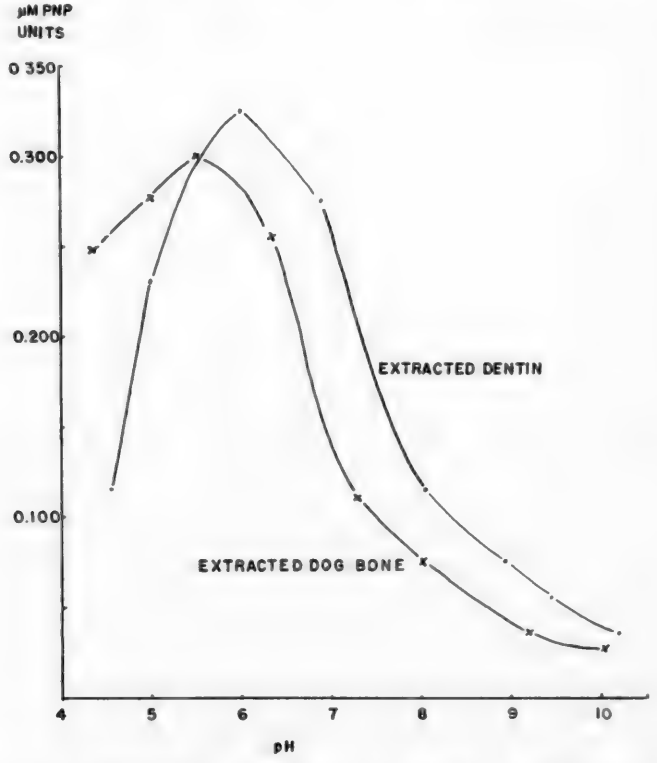

Fig. 4.—pH-"phosphatase" Activity Curve of Extracted Human Dentin and Extracted Dog Bone. Each point indicates the activity in μM PNP units of 100 mg. of the ethylene diamine-extracted dentin or bone powder, at that pH. Each reaction vessel contained 0.1 per cent PNPP, 0.00625 M glycine, 0.01 M acetate-barbital or carbonate-barbital buffer, and 0.01 M $MgCl_2$, in a total volume of 4.0 ml. Incubated 1 hour at 37° C.

The residue was centrifuged and washed 3 times with distilled water. A 1 per cent solution of $MgCl_2$ was then added to the residue and placed in a

refrigerator for 10 hours. The protein was again centrifuged and resuspended in 5.0 ml. of distilled water. The suspension was assayed for alkaline phosphatase activity, at pH 10.0, with the amount of protein corresponding to 100 mg. of the original dentin, 0.015 μM PNP units was obtained. The activity of the protein is 23 per cent of the original activity of the dentin. Examination of Table I shows that heating dentin reduces its activity at pH 10.1 by 43 per cent, if this decrease in activity is a measure of the enzymatic activity then about one half of the possible enzymatic activity was recovered in the protein remaining after decalcification.

Respiratory Enzymes.—Pincus[4] described what is, apparently, a peroxidative reaction for the detection of "oxidase"; the addition of an alcoholic solution of alpha naphthol and hydrogen peroxide to a suspension of dried root dentin in water produced a lavender color. When this procedure was applied to 200 mg. samples of root dentin, we were unable to observe color formation. Since gum guaiac is an indicator of oxidase or peroxidase activity 5 drops of a 1.5 per cent alcoholic solution of guaiac were added to a similar dentin suspension, no blue color formed and the addition of 3 per cent hydrogen peroxide solution likewise produced no color. The Nadi reaction for indophenol oxidase using alpha-naphthol and p-dimethylphenylene diamine solutions, was also negative. No evidence could be obtained for the presence of an oxidase in any sample of fresh root dentin.

The method of Friedemann and Hollander[22] was adapted for the assay of dehydrogenase activity in dentin. In this technique the reduction of methylene blue is not carried out in vacuo, rather the dye is dissolved in agar and then the solidified agar is exposed to the air. Oxygen from the air will diffuse into the top layer of the agar, reoxidizing the leukomethylene blue formed by the dehydrogenase, however reduction may still be observed in the lower part of the tube. The procedure described by Pincus was followed, after 90-minute incubation there was no detectable color difference in any of the tubes. Incubation for an additional 16 hours still failed to produce color changes. Several repetitions of this experiment with different samples of fresh root dentin all gave negative results.

The classical Thunberg method for dehydrogenase assay[23] was likewise used. In each of 2 Thunberg tubes was placed 200 mg. of root dentin and 2.0 ml. of M/15 phosphate buffer, pH 7.0. In the sidearm cap of the first tube, and in the body of the second tube, was placed 0.1 ml. of 1:10,000 methylene blue solution and 0.1 ml. of 0.1 M sodium succinate solution. Tube two was evacuated for 5 minutes and then both tubes were placed in a constant temperature bath at 37° C. for 3 hours. At the end of that time the contents of the sidearm cap of tube one was tipped into the tube and the colors of the tubes compared, no appreciable difference was observed. This experiment was repeated with 2 different samples of fresh root dentin with the same result. The reaction mixture was reduced by a yeast suspension.

Blue tetrazolium can be reduced by dehydrogenase systems forming a highly colored compound. The reduced form of the dye is not reoxidized by

atmospheric oxygen[24] and may be used, therefore, without evacuation of the reaction vessel. Blue tetrazolium was used by Seligman and Rutenberg[25] for the histochemical demonstration of succinic dehydrogenase. An adaptation of this method was employed in the following manner; 1.0 ml. of a 0.001 per cent aqueous solution of blue tetrazolium and 1.0 ml. of 0.1 M phosphate buffer, pH 7.6, were added to each of 4 test tubes. To 2 of the tubes was added 1.0 ml. of 0.2 M sodium succinate and all tubes were then made up to 4.0 ml. with distilled water, 100 mg. of root dentin was then added to one of the tubes containing succinate and also to one of the tubes not containing succinate. The 4 tubes were stoppered, shaken, and incubated at 37° C. for 3 hours. There was no visible reduction in any tube. A small piece of frog muscle added to the succinate reaction mixture showed a distinct blue color within 30 minutes.

As an additional check a freshly extracted molar was cleaned and cut in half longitudinally with a separating disk, exposing the pulp chamber. One half of the tooth was incubated in the tetrazolium-succinate reaction mixture for 90 minutes at 37° C. and compared with the untreated half. The pulp was stained blue but the dentin, enamel, and cementum showed no color.

DISCUSSION

No soluble phosphatase could be isolated from dentin, there was evidence, however, for the presence of a small amount of a bound phosphatase. The fact that some activity was obtained with decalcified dentin suggests the association of this phosphatase with the dentinal collagen that has been shown to adsorb phosphatase.[6] The close association of the activity with the insoluble collagen also explains the inability to prepare an active extract from dentin. The mature bone powders and the dentin had considerable bound alkaline phosphatase activity that was destroyed by heat. The phosphatase was not extractable from the mature bone powders and it is probable that the phosphatases of dentin and bone are analogous.

The nonenzymatic "phosphatase" activity of dentin is probably due to the inorganic phase since it is detectable after the organic matrix is removed. The pH-activity curve of the extracted dentin shows a shift in the pH optimum to the left when compared with the curve given by unextracted dentin, the extraction process may well produce changes in the crystal structure of the inorganic phase.

When the organic matrix of bone powder was extracted a pH-activity curve was obtained that was quite similar to that of extracted dentin and markedly different from the activity of the original bone. Activity in the pH range 5 to 6 was slight in the original bone, but in the extracted bone it was increased while the activity near pH 10, which was high in the original bone, is eliminated by extraction. Bamann and Meisenheimer[26] found that rare earth metal hydroxide gels in aqueous suspension catalyze the hydrolysis of phosphate esters, maximum activity was obtained from pH 8 to 9. The present work shows that the mineral phase of dentin and bone also catalyzes

phosphate ester hydrolysis. A calcium phosphate gel likewise showed activity, it is not possible at this time to assess the importance of these observations to the theory of calcification, further studies on the hydrolysis of physiologically important phosphate esters should be made.

CONCLUSIONS

A soluble alkaline phosphatase could not be isolated from human dentin or the mature bones of the dog and the monkey. A bound alkaline phosphatase could be demonstrated in both dentin and bone which was associated with the organic phase. Activity of a phosphatase-like nature could be obtained from dentin and bone following the removal of the organic phase. This activity had a different pH optimum than that of the unextracted material and is probably due to a catalytic activity of the inorganic phase.

Previous reports of the presence of respiratory enzymes in human coronal dentin could not be confirmed.

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OBSERVATIONS ON SEXUAL SIMILARITY AND BILATERAL DISTRIBUTION IN DENTAL CARIES INCIDENCE IN ALBINO RATS AND COTTON RATS

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CONSIDERABLE evidence has been presented over the years to indicate a higher incidence in dental caries attack rate and in total accumulated caries experience in the permanent teeth of women than in men at comparable ages. These data have been reviewed in detail recently by Finn.[1] This observation may be partially or completely explained by the earlier eruption of the teeth in girls with the resultant greater posteruptive tooth age in girls than in boys of the same age.[2-5] Among laboratory animals, the male hamster has been shown by Dale and Keyes[6] and Keyes[7, 8] to be much more susceptible to tooth decay than littermate females of the same age and experimental conditions. This difference in caries-susceptibility between sexes of the hamster has been repeatedly confirmed by American investigators and is far greater than the difference in caries incidence between men and women of the same age. However, Granados, Glavind, and Dam,[9] and Gustafson, Stelling, and Brunius[10] failed to detect any difference in dental caries incidence between the sexes in the hamster. The dental caries experience of the hamsters in the experiments reported by Granados, Glavind, and Dam[9] was sufficiently low that there is reasonable doubt as to whether a sound comparison could be made between the sexes. However, in the case of the data presented by Gustafson, Stelling, and Brunius,[10] the dental caries experience of the groups reported was in the same range as that reported by Keyes and co-workers.[6-8] Elsewhere Keyes[11] has pointed out that the sex difference in hamsters is best demonstrated when the animals are sacrificed sufficiently early so that none of the groups have attained the maximum degree of tooth destruction. When the animals are sacrificed after this optimum time, the caries experience of the females has had an opportunity to continue to increase after the maximum destruction has occurred in the males. Thus any difference between the sexes can be decreased or even obscured.

Bilateral symmetry in dental caries incidence has been widely accepted by investigators both in clinical and laboratory trials. Wheatcroft, Gerende, Schlack, Taylor, Berzinskas, and Mullins[12] have presented a thorough review of the clinical literature and described an experiment in rats where a relatively high degree of bilateral symmetry was observed. However, they aptly drew attention to the fact that there was by no means a perfect bilateral symmetry and that the assumption of complete bilateral symmetry should not

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be made in experimental comparisons. This was indicated to be particularly true where the data for individual animals were being compared instead of averages for groups of animals.

In order to explore the relative incidence of tooth decay in males and females and the degree of bilateral symmetry in albino rats and cotton rats, a survey was conducted of the dental caries experience in all suitable representatives of these two species which had been on experiment in this laboratory during the 9 years prior to April 1, 1954.

EXPERIMENTAL

The subjects surveyed in this study were of 2 species, the albino rat (*Mus norvegicus*) and the cotton rat (*Sigmondon hispidus hispidus*). All subjects were offspring of the stock colonies of these 2 species which have been used in this laboratory for dental caries research. The animals for which dental caries experiences are reported had not been maintained purely for this study but instead were selected from all animals which had been used in dental caries experiments in this laboratory. The selection was based on the ability to choose from any suitable experimental treatment at least one male and one female which were littermates and which had been maintained for the same period. Within each species, both caries-susceptible and caries-resistant strains were represented to varying degrees. No specimens were included from groups in which the health and well-being of the animals had been appreciably impaired. Likewise, no treatment was included where any effort had been made to alter the caries incidence of one side of the head but not the other side, or where any surgical manipulation of the testes or ovaries was undertaken. Hence, the various animals in these groups had been maintained on any one of several dozen different rations and experimental regimens and had been on experiment for different periods of time. In all cases, the diets used were modifications of the highly purified diet described by Shaw.[13]

In the course of this selection process 709 male and 692 female albino rats and 688 male and 626 female cotton rats were judged to be highly suitable for the comparison of the caries incidence in male versus female rodents. The same animals in their entirety were used for the bilateral comparisons of dental caries incidence. Throughout the respective experimental periods, all animals were housed in individual wire-bottom cages and fed ration and tap water ad libitum. The usual means of scoring caries lesions used in this laboratory was employed throughout the entire period of these trials.[14]

RESULTS AND DISCUSSION

The comparisons of the average number of carious teeth, the average number of carious molars and the average extent of carious lesions for male and female rodents of both species are presented in Table I. Regardless of which of the 3 criteria of dental caries incidence was used for the comparison, no significant difference was noted between the male and female representatives of either species. In addition to the comparisons made for the total groups as presented previously a subdivision into caries-resistant and caries-susceptible populations was made. Again no difference in caries incidence was observed between the sexes among either the caries-resistant or the caries-susceptible members of the 2 species. These data suggest that, unlike the hamster, the two sexes of the albino rat and of the cotton rat have comparable caries susceptibilities. In these species, this observation applies whether the rate of initiation and progression of carious lesions is high or low.

TABLE I
A COMPARISON OF THE DENTAL CARIES INCIDENCE IN MALE AND FEMALE ALBINO RATS AND COTTON RATS

SPECIES	NO. OF SPECIMENS	SEX	NUMBER OF CARIOUS MOLARS			NUMBER OF CARIOUS LESIONS			EXTENT OF CARIOUS LESIONS		
			AV.	S.E.M.*	C.R.†	AV.	S.E.M.*	C.R.†	AV.	S.E.M.*	C.R.†
Albino rat	709	♂	3.9	0.2	0.4	5.0	0.3	0.4	10.9+	0.9+	0.7
	692	♀	4.0	0.2		5.2	0.4		12.1+	1.4+	
Cotton rat	688	♂	9.1	0.3	0.2	23.1	0.4	0.4	66.2+	1.5+	1.5
	626	♀	9.0	0.3		22.9	0.4		63.0+	1.5+	

*Standard error of mean.
†Critical ratio: The ratio of the difference between 2 means to the standard error of the difference between the means. Whenever the critical ratio is less than 2.0, the difference between the means is considered statistically insignificant. The diagonal lines in the table connect sets of data which are being compared for the critical ratio test of significance.

The over-all comparisons of the incidence of dental caries on the left and right sides of the head of the albino rat and the cotton rat are presented in Table II. Thus, when large groups of representatives of both species are compared, a highly similar incidence of tooth decay was observed on the 2 sides of the head. However, this is not a true index of the actual degree of bilateral distribution of carious lesions. The same data were compared to determine how frequently there was a bilateral pairing of the carious lesions. In the 1,314 cotton rats, 92 per cent of the carious lesions occurred in bilaterally symmetrical pairs. Among the 1,401 albino rats, 83 per cent of the lesions

TABLE II
DISTRIBUTION OF CARIOUS LESIONS ON THE LEFT AND RIGHT SIDES OF ALBINO RATS AND COTTON RATS

SPECIES	NO. OF SPECIMENS	SIDE	NUMBER OF CARIOUS MOLARS			NUMBER OF CARIOUS LESIONS			EXTENT OF CARIOUS LESIONS		
			AV.	S.E.M.*	C.R.†	AV.	S.E.M.*	C.R.†	AV.	S.E.M.*	C.R.†
Albino rat	1,401	Left	2.0	0.1	0	2.6	0.1	0.1	6.0+	0.5+	0.4
		Right	2.0	0.1		2.5	0.1		5.7+	0.4+	
Cotton rat	1,314	Left	4.5	0.1	0	11.5	0.1	0	32.3+	1.6+	0.1
		Right	4.5	0.1		11.5	0.1		32.4+	1.7+	

*Standard error of mean.
†Critical ratio.

were bilaterally paired. It was interesting again to regroup the animals according to caries-susceptibility. The caries-susceptible groups in both species displayed a much higher degree of bilateral symmetry than the caries-resistant groups. In the caries-susceptible group of white rats, bilateral pairing of carious lesions amounted to 90 per cent in comparison to 68 per cent for the caries-resistant group. Bilateral pairing was observed for 96 per cent of the lesions in the caries-susceptible group of cotton rats, in contrast to 84 per cent for the caries-resistant group. Highly resistant animals in both species frequently showed bilateral pairing at a ratio as low as 50 per cent of the total number of lesions. In the most susceptible animals of both species, perfect bilateral pairing was commonly observed. The latter observation occurred especially frequently in the cotton rat.

Just as bilateral symmetry of carious lesions was greatest in the caries-susceptible animals, a comparable observation was made for the most susceptible pairs of grooves and the most susceptible teeth within any mouth. Uniformly, the most caries-susceptible pairs of grooves and the most caries-susceptible pairs of teeth displayed a higher degree of bilaterally symmetrical development of carious lesions than did the more resistant pairs of grooves and teeth. This relationship was also observed in the groups of albino rats studied by Wheatcroft, Gerende, Schlack, Taylor, Berzinskas, and Mullins.[12]

SUMMARY

The caries-susceptibility of males and females has been compared in 1,401 albino rats and 1,314 cotton rats. In addition, the degree of bilateral symmetry in the distribution of carious lesions among the same experimental subjects has been determined.

No difference in caries-susceptibility was observed between males and females of these two species. This similarity between the sexes was noted among both caries-susceptible and caries-resistant groups.

A high degree of bilateral symmetry was observed in the distribution of carious lesions in these species. However, this degree was correlated with the caries-susceptibility of the individual animals, teeth and grooves. Bilateral symmetry was greater in caries-susceptible groups of rodents than in the caries-resistant groups. Bilateral symmetry was also greater in the more susceptible pairs of grooves and teeth than in the more resistant pairs.

The rodents in this survey were from various experiments conducted to a large extent with the support of funds from the National Biscuit Company, New York, the Nutrition Foundation, Inc., New York, the Sugar Research Foundation, Inc., New York, and the Division of Research Grants and Fellowships, U. S. Public Health Service.

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A COMPARATIVE STUDY OF ORAL LACTOBACILLI ISOLATED ON TWO DIFFERENT MEDIA FROM THE HUNT-HOPPERT RATS

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IT HAS been shown that lactobacilli are recovered more frequently, and in greater numbers, from caries-susceptible than from caries-resistant rats of the Hunt-Hoppert strains.[1-3] Since both tomato juice agar special (Difco) and lactobacillus-selection medium (BBL) are used in this laboratory to isolate and enumerate oral lactobacilli, it was decided to compare the types of lactobacilli appearing on each of these media as well as the types recovered from caries-resistant and caries-susceptible rats.

In contrast with human oral lactobacillus types, very little has been published about the various rat oral lactobacilli. Fitzgerald, Jordan, and Zipkin[4] expressed the belief that rat oral lactobacilli differ from human oral lactobacilli in several biochemical and nutritional characteristics.

Although no species names were assigned to the lactobacilli investigated in this study, it is believed that sufficient data are presented to illustrate the importance of rat oral lactobacilli in the taxonomy of this group of bacteria.

EXPERIMENTAL METHODS

Isolation of Strains.—The bacteria surrounding the teeth were obtained by brushing the lower right molars with a cotton swab moistened in 1.0 per cent peptone water. The swab was broken off at the tip into 5.0 ml. of 1.0 per cent peptone water. Appropriate dilutions of this suspension were made and plated on the surface of tomato juice agar special (Difco) containing 0.02 per cent sodium azide, and on lactobacillus-selection medium (BBL).

The rats were maintained on the HWC diet.[5, 6] Bacterial colonies were selected for study only if 30 or more of one type appeared on the same plate. Only one colonial type per plate was chosen.

Originally a comparison was to have been made between lactobacilli isolated from caries-resistant and caries-susceptible rats as well as lactobacilli isolated from the two media. When 15 strains from caries-resistant rats isolated from lactobacillus-selection medium and 22 strains isolated from tomato juice agar were found to be very similar to lactobacilli isolated from caries-susceptible rats on these two media, respectively, the rest of the strains were secured from caries-susceptible animals in order to hasten the collection of organisms. A total of 200 strains was obtained; 100 from each of the media.

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The 200 strains were replated at least once, and more often if necessary, to ensure the establishment of a pure culture by noting the reappearance of the same colonial type. It was easier to describe the colonial type appearing on lactobacillus-selection medium than on tomato juice agar because the colonies were 2 to 3 times larger. It was often difficult to ascertain purity of the strains isolated from tomato juice agar. In those cases, the strains were replated twice and used for further study.

Colonies selected for study were transferred into 4 ml. of micro inoculum broth (Difco). They were maintained in stab culture in micro assay culture agar (Difco) and were transferred monthly.

The following preliminary procedures were carried out: gram stain employing the Hucker modification, catalase test, and tests for the reduction of nitrate to nitrite. The gram stain and catalase tests were made after 24 hours' incubation at 37° C. in microinoculum broth. The test for the reduction of nitrate to nitrite was made in indole-nitrite medium (BBL) after 3 days' incubation at 37° C. Before concluding that an organism did not reduce nitrate to nitrite, a test for residual nitrate was performed by addition of zinc dust, and good growth was noted in the tubes. Only gram-positive, catalase-negative rods were retained for further study. All the organisms tested for the reduction of nitrate to nitrite were negative except one. It was retained for further study when Costilow and Humphreys[7] found that certain lactobacilli gave positive results when tested in indole-nitrite medium.

Although nearly all the organisms from lactobacillus-selection medium were gram-positive, catalase-negative rods, only about 80 per cent of the organisms from tomato juice agar would fit this description. The other 20 per cent were gram-positive cocci. Although lactobacillus-selection medium is more selective for lactobacilli than tomato juice agar special, we have recovered lactobacilli more frequently and in greater numbers from the latter medium.

Fermentation Tests.—Fermentation tests were conducted in the following basal medium which was patterned after microinoculum broth: yeast extract (Difco), 20 Gm.; proteose peptone No. 3 (Difco), 5 Gm.; Tween 80, 1 Gm.; bromcresol purple, 0.015 Gm.; distilled water, 1 L.; pH, 6.9. The following substrates were used: L-arabinose 1.0 per cent, D-glucose 1.0 per cent, dextrin 1.0 per cent, D-lactose 1.0 per cent, D-sucrose 1.0 per cent, D-raffinose 0.5 per cent, D-mannitol 1.0 per cent, salicin 0.5 per cent. The media were dispensed in 4 ml. quantities into 12 by 100 mm. test tubes. Arabinose was sterilized at 115° C. for 12 minutes.

The inocula were prepared by transferring from the stock culture into 4 ml. of microinoculum broth. When abundant growth was observed (generally 24 hours) a second broth transfer was made. When abundant growth was observed in the second transfer, the entire content of the tube was poured into 20 ml. of distilled water. One drop of this suspension was used as the inoculum. The inocula were used immediately after preparation. Generally abundant growth was observed after the second transfer within 24 hours. If after

72 hours an organism had not attained abundant growth, it was used if there was any growth at all. The indicator was observed daily for 2 weeks and a notation was made the day it changed color distinctly.

Litmus Milk.—Four milliliters of litmus milk was dispensed into 12 by 100 mm. tubes. They were inoculated as described in the fermentation tests and incubated for 2 weeks at 37° C. Observations were made daily for changes in the indicator and for any change in the physical character of the milk.

Test for Gas Production.—One drop of an active 24-hour culture was inoculated into 10 ml. of microassay culture agar which had been previously melted and held at 45° C. After solidification, the medium was layered with nonnutrient 2 per cent agar, which was subsequently layered with mineral oil. The tubes were incubated at 37° C. and observed daily for 7 days for cracks in the agar or any other signs of gas production.

pH.—The organisms were tested for their effect on the pH of microinoculum broth which contains the sugar dextrose in a concentration of 1 per cent. Ten milliliters of the broth was dispensed into 16 by 150 mm. test tubes. They were inoculated as previously described and incubated for 72 hours at 37° C., after which the pH was determined electrometrically.

Growth on Synthetic Medium With and Without Tween 80.—Medium I of Sauberlich and Baumann[8] with and without Tween 80 was used. The concentration of Tween 80 was 0.1 per cent. Four milliliters was dispensed into 12 by 100 mm. test tubes and inoculated as in the fermentation tests. After 3 days' incubation at 37° C., growth was observed visually. Designations as to the amount of growth were made as follows: – = no growth, 1+ = doubtful growth, 2+ = slight growth, 3+ = moderate growth, 4+ = abundant growth.

Colonial Characteristics.—The colonial characteristics of the 200 strains were studied on each of the 2 media. It proved too difficult consistently to separate the strains on the basis of their colonial characteristics on tomato juice agar. The description of colonial characteristics could not be made more certain when ordinary tomato juice agar was substituted for the tomato juice agar special containing 0.02 per cent sodium azide. However, the colonial characteristics of the organisms on lactobacillus-selection medium were easily studied due to the larger size of the colony.

The inocula for this study were prepared as described in the fermentation tests except that after abundant growth was observed in the second transfer a sterile swab was immersed into the broth culture and streaked onto each of the 2 media. A second, sterile swab was used to thin out the original streak. After 3 days' incubation at 37° C., the colonies were observed under ×20.7 magnification. A colony was deemed smooth if it was opaque, had an entire edge, and was at least 1.0 mm. in diameter. All other colonies were considered irregular.

Repetition of Tests.—All the tests were repeated. If a result was variable, the test was redone twice. In the fermentation and litmus milk tests, a result

TABLE I
CHARACTERISTICS OF RAT ORAL LACTOBACILLI ISOLATED FROM LACTOBACILLUS-SELECTION MEDIUM (BBL)

Group	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
No. Strains	78	4	3	3	2	1	1	1	1	1	1	1	1	1	1
Arabinose	+1	-	-	-	-	+3	-	-	-	-	+3	-	+1	+8	-
Glucose	+1	+2	+6	+1	+4	+3	+3	+4	+2	+1	+1	+1	+1	+4	+4
Dextrin	-	-	-	+1	-	-	+5	-	-	+3	+1	-	+1	-	-
Lactose	+2	+5	-	-	-	+9	+1	+5	+9	+1	+1	+1	+1	-	+3
Sucrose	+1	+3	-	+1	+3	+4	+1	+2	-	+1	+1	+1	+1	±	+3
Raffinose	+2	+4	-	-	-	+2	+1	-	-	-	-	+1	+1	±	-
Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	+1	-	-
Salicin	-	-	-	+3	-	-	+1	-	-	+2	+3	+1	+1	-	-
Litmus Milk	10 A or NC	NC	NC	NC	NC	NC	A^1C^2	NC	NC	$A^8C^{\pm}$	A^5C^9	A^2C^3	NC	NC	NC
Colony on lactobacillus-selection medium	S	I	I	I	I	I	I	I	I	I	I	I	I	I	I
pH	5.0	6.0	6.0	4.6	6.3	6.2	4.6	6.0	6.1	4.5	4.5	4.7	5.9	6.2	6.1
Growth in Sauberlich and Baumann medium	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Growth in Sauberlich and Baumann medium + Tween 80	4+	3+	3+	2+	2+	2+	2+	1+	2+	-	-	-	1+	-	4+

Superscripts signify day at which positive reaction was first noted.
A = acid; C = curd; NC = no change; S = smooth colonies; I = irregular colonies; ± = weak or variable reaction.

was considered variable if there was greater than a 7-day difference in change in the indicator or physical character of the milk. A tolerance of ±0.1 pH unit was allowed in the determination of pH in microinoculum broth.

Use of Controls.—Whenever a series of tests was performed, an equal number of strains isolated from each of the 2 media were used. The inoculated tubes in the fermentation and litmus milk tests were always compared with uninoculated tubes held in the incubator. This precaution had to be taken especially with arabinose. In spite of reduced heat used in sterilizing this sugar, an uninoculated tube would generally turn yellow after 7 days' incubation, but tubes kept in the refrigerator retained their purple color for several weeks.

RESULTS

The results are presented in Tables I and II. The values given in the tables are averages of data which varied slightly. The strains isolated from lactobacillus-selection medium differed very distinctly from the strains isolated from tomato juice agar. On the basis of tests conducted thus far, 78 per cent (Table I, Group 1) of the strains from lactobacillus-selection medium were identical. Only 6 per cent (Table II, Group 6) of these strains were isolated from tomato juice agar. The tomato juice agar strains generally fermented dextrin and salicin whereas the lactobacillus-selection medium strains rarely fermented those substrates. There was only 1 strain from lactobacillus-selection medium which fermented mannitol, whereas there were 23 from tomato juice agar. More strains from tomato juice agar than from lactobacillus-selection medium curdled milk as well as reduced the pH of microinoculum broth to a lower level.

It is interesting to note that very few strains grew in the synthetic medium of Sauberlich and Baumann unless Tween 80 was added. Even with the addition of Tween 80, comparatively few strains from tomato juice agar grew well whereas nearly all the strains from lactobacillus-selection medium exhibited moderate or abundant growth.

Perhaps the most significant difference between the organisms isolated from the 2 media was that 64 per cent of tomato juice agar stains failed to grow on lactobacillus-selection medium, whereas nearly all the strains from lactobacillus-selection medium grew on tomato juice agar. Twenty-eight per cent of the tomato juice agar strains developed an irregular colony on lactobacillus-selection medium and only 8 per cent produced the typical smooth colony associated with the medium.

Clear-cut results were not obtained when the strains were tested for gas production. The few strains that did produce gas, generally produced a minute amount although growth was observed in the tube. Under the conditions of this test, it appears that most of the strains from both media were homofermentative.

DISCUSSION

Rat oral lactobacilli are influenced by controlled conditions such as diet, temperature of animal house, the lineage of the animals, etc., whereas these

TABLE II. CHARACTERISTICS OF RAT ORAL LACTOBACILLI

Group No. Strains	1 22	2 14	3 13	4 12	5 7	6 6	7 4	8 4	9 2	10 2
Arabinose	-	+1	+1	-	-	+1	+1	-	-	-
Glucose	+1	+1	+1	+1	+1	+1	+1	+1	+6	+1
Dextrin	+4	+6	+7	+2	+3	-	-	-	-	+6
Lactose	+1	+1	+1	-	+1	+1	+3	±1	-	-
Sucrose	+1	+1	+1	+4	+1	+1	+2	+1	-	+8
Raffinose	+2	+1	+1	-	-	+2	+3	+2	-	-
Mannitol	-	+5	-	-	-	-	+3	-	-	-
Salicin	+1	+1	+1	+4	+4	-	-	+1	-	-
Litmus Milk	A^1C^2	A^{12}	A^{12}	NC	A^7C^{11}	A^7 or NC	A^{13} or NC	A^2C^3	NC	NC
Colony on lactobacillus-selection medium	NG*	NG	NG	I NG	NG*	S	NG	NG	NG†	I
pH	4.6	4.7	4.7	4.5	4.5	5.0	5.1	4.6	5.9	4.5
Growth in Sauberlich and Baumann medium	-	-	-	-	-	-	-	-	-	-
Growth in Sauberlich and Baumann medium + Tween 80	-	-	-	-	-	4+	4+	-	-	1+

Superscripts signify day at which positive reaction was first noted.

N = nitrate positive; A = acid; C = curd; NC = no change; NG = no growth; S = smooth colonies; I = irregular colonies; ± = weak or variable reaction; * = 5 strains grew; colonies were irregular; † = 1 strain grew; colonies were irregular.

conditions are not controlled in humans and thereby for the lactobacilli in humans. Consequently, types of oral lactobacilli obtained from rats subject to different environmental and genetic factors may quite likely possess different characteristics. However, from the results of this investigation several points may be emphasized concerning the study of lactobacilli.

When 2 or more selective media are used to isolate this group of bacteria, different types may be obtained. Tilden and Svec[9] have also pointed out that they were able to obtain *Lactobacillus acidophilus* only from tomato agar and not from DST and Snyder's medium.

Most of the strains isolated from tomato juice agar failed to grow on lactobacillus-selection medium. This may not be too surprising considering the effectiveness with which this medium inhibits the closely related oral streptococci. One cannot be too certain what new strains may be inhibited by selective media. Perhaps this may explain why lactobacilli are not isolated from some individuals with dental caries.

There is a definite growth response of certain strains of oral lactobacilli to Tween 80 especially those isolated from lactobacillus-selection medium. This is not too unusual since this medium as well, as most of the other media used in the study of these bacteria, contained this compound. Probably these organisms require a low surface tension. Benarde[10] has shown that the saliva of both the caries-resistant and caries-susceptible rats has a surface tension ranging from 42.3 to 45.1 dynes per centimeter.

SOLATED FROM TOMATO JUICE AGAR SPECIAL (DIFCO)

11	12	13	14N	15	16	17	18	19	20	21	22	23
2	1	1	1	1	1	1	1	1	1	1	1	1
+1	+1	–	+2	–	–	+3	–	–	–	–	–	–
+1	+1	+1	+2	+1	+1	+1	–	+1	+1	+1	+4	+5
+3	+1	+1	–	+3	+8	–	–	+8	+5	–	+4	–
+1	+1	–	+3	+1	+2	+1	–	+1	–	+5	–	–
+1	+1	+1	+2	±1	–	±1	–	+1	–	+2	–	–
+2	+4	–	+4	–	–	+2	–	+1	–	+7	–	–
+2	+1	–	+3	–	–	–	–	+4	–	–	–	–
+1	+1	+1	–	+3	–	+1	–	+1	–	–	–	–
A^4C^6	A^1C^1	A^1C^1	NC	A^8	A^2C^2	A^1C^2	NC	A^5	NC	NC	NC	A^8C^{12}
NG†	NG	I	NG	NG	S	NG	NG	NG	I	S	NG	NG
4.8	4.8	4.6	5.2	4.5	4.7	4.6	6.3	4.7	4.5	5.1	5.6	4.7
–	2+	–	–	–	1+	–	–	–	1+	–	–	–
4+	2+	–	–	4+	1+	1+	1+	–	2+	4+	3+	–

There was very little variation in the characteristics of individual strains when the tests were repeated. The principles set forth by Rogosa and associates[11] of using nutritionally adequate media and an active culture were adhered to throughout this study. Considerable variation was experienced before we used nutritionally adequate media.

The more frequently occurring strains are being retained for further study and are available on request.

SUMMARY

A comparative study has been made on 100 strains of rat oral lactobacilli isolated from lactobacillus-selection medium and an equal number of strains isolated from tomato juice agar. In contrast with lactobacilli isolated from tomato juice agar, most of the strains from lactobacillus-selection medium appeared to be of 1 type, generally did not ferment dextrin or salicin, and only 1 strain fermented mannitol. These organisms rarely curdled litmus milk and did not reduce the pH of a dextrose broth medium as much as strains from tomato juice agar. These strains grew well in a synthetic medium containing Tween 80 whereas the growth of organisms isolated from tomato juice agar was sparse in the same medium. The majority of tomato juice agar lactobacilli did not grow on lactobacillus-selection medium which indicates that a medium designed to select a group of organisms may be inhibitory to certain strains.

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ADDENDUM

After this article was submitted for publication, it was learned that lactobacillus-selection medium (BBL) should not be used for surface plating. When pour plates were made of rat oral microflora in this medium, about 10 times as many lactobacilli were recovered as when surface plated. However, the ratio of the types of organisms with which this study was primarily concerned was approximately the same. About 5 to 10 per cent more irregular colonies were obtained. Organisms from tomato juice agar, which did not grow on the surface of lactobacillus-section medium, grew in subsurface culture producing an irregular colony.

THE PRODUCTION OF CHONDROSULFATASE BY MICROORGANISMS ISOLATED FROM HUMAN GINGIVAL CREVICES

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AS part of an investigation of the possible functions of bacteria in the development of chronic marginal gingivitis and periodontal disease, we have reported the production of hyaluronidase and beta-glucuronidase[1] and phenolsulfatase[2] by gingival microorganisms. In addition to hyaluronic acid, chondroitin sulfate is among the components of gingival tissue,[3, 4] forming a part of the intercellular "cementing" substances. It was, therefore, pertinent to determine whether or not gingival microorganisms produce an enzyme, capable of catalyzing the breakdown of this substance, by means of which they might contribute to the structural disintegration of the gingivae.

The enzyme, chondrosulfatase (chondroitin sulfatase, chondroitinase),[5, 6] catalyzes the hydrolysis of the mucopolysaccharide, chondroitin sulfate. Neuberg and Rubin[7] established in 1914 that inorganic sulfate is formed from chondroitin sulfate by putrefactive bacteria. Later reports have shown that some strains of *Proteus vulgaris, Pseudomonas aeruginosa,* and a microorganism related to *Pseudomonas eisenbergii* produce chondrosulfatase.

An indication of the presence of chondrosulfatase in cell-free extracts of gingival accumulations has been reported previously.[8] The present paper describes the production of this enzyme by bacteria isolated from gingival crevices and pockets.

MATERIALS AND METHODS

Beef-extract broth: Preliminary trials showed that the broth used for the bacterial production of phenolsulfatase was satisfactory also for the production of chondrosulfatase. The following recipe was used:

Beef extract (Difco)	10 Gm.
Peptone (Difco)	10 Gm.
Yeast extract (Difco)	2.5 Gm.
Sodium chloride (reagent grade)	5 Gm.
Distilled water	1,000 ml.

The pH was adjusted to pH 7.2 and the medium was sterilized in an autoclave at 120° C. for 20 minutes.

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Mixed cultures: Subgingival accumulations were collected as described previously[1] and inoculated immediately into test tubes containing 10 ml. of beef-extract broth. The tubes were incubated aerobically for 48 hours at 37° C.

Pure cultures: Samples of the above mixed cultures were streaked out on thioglycollate agar, sheep blood agar, and Bordet-Gengou agar (with sheep blood) plates, which were incubated aerobically (in closed containers to prevent drying) and anaerobically in a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide for from 1 to 6 days at 37° C. Single colony strains of the various types that grew were isolated on the same media in the conventional manner. As a guide to the search for microorganisms producing chondrosulfatase, it was deemed adequate to characterize them by their colonial appearance and by the microscopic examination of gram-stained smears. After isolation, aerobic, and anaerobic cultures of these organisms in the beef-extract broth were tested for chondrosulfatase.

Substrate: Chondroitin sulfate was prepared from bovine nasal cartilage by the method of Einbinder and Schubert.[9]

Assay procedure: The hydrolysis of chondroitin sulfate has been measured previously by determining the liberation of inorganic sulfate, of ammonia, of reducing sugars, and of hexosamine.[10-13] For the purpose of the present investigation, it was deemed more relevant to determine simply the degree of depolymerization, for this is a measure of the loss of the ability of the mucopolysaccharide to function as a tissue "cementing" substance. Accordingly, a combination was used of the turbidimetric methods of Tolksdorf, McCready, McCullagh, and Schwenk[14] for the assay of hyaluronidase, and of Mathews and associates[5] for the determination of the chondrosulfatase activity of bovine testicular preparations. Like hyaluronic acid, chondroitin sulfate forms insoluble complexes in vitro with proteins in acid solution. Under standard conditions, a given amount of chondroitin sulfate gives a reproducible turbidity when added to a standard amount of protein, and the turbidity is proportional to the amount of chondroitin sulfate. If the polysaccharide be depolymerized, it no longer combines with proteins and the turbidity of test mixtures is decreased proportionately. The ability of a bacterial culture to bring about such a depolymerization is a measure of its chondrosulfatase content.

The procedure finally adopted was as follows: A standard solution was prepared containing 0.25 mg. of chondroitin sulfate per ml. in a phosphate-citrate buffer at pH 5.0 (9.70 ml. of 0.1 M citric acid plus 10.30 ml. of 0.2 M Na_2HPO_4). Bacteria were removed from the broth cultures by centrifugation. One milliliter of an appropriate dilution of supernate was added to 1 ml. of the standard solution of chondroitin sulfate in a Klett-Summerson colorimeter tube. Several drops of chloroform were added to prevent secondary bacterial growth. The mixtures were incubated for 18 hours at 37° C. (A series of tests showed that with critical dilutions of the enzyme, that is, those producing only partial breakdown of the substrate, hydrolysis of the chondroitin sulfate reached its maximum and ceased within 6 hours. The

18-hour period was chosen merely for convenience.) After incubation, the amount of unhydrolyzed chondroitin sulfate was determined by adding to each tube 1 ml. of acidified, heat-stabilized human plasma[14] plus 2 ml. of 0.5 M acetic-acid sodium-acetate buffer at pH 4.2 in order to bring the total volume to the 5 ml. required by the colorimeter. Ten minutes were allowed for the development of turbidity, which was measured in a Klett-Summerson photoelectric colorimeter, using red filter No. 66. The amount of chondroitin sulfate was calculated by reference to a standard curve determined with mixtures of from 0.50 to 0.010 mg. of chondroitin sulfate in 1 ml. of the pH 5.0 phosphate-citrate buffer, plus 1 ml. of the beef-extract broth, 1 ml. of the acidified plasma solution, and 2 ml. of the pH 4.2 acetate buffer.

One unit of chondrosulfatase was defined as the amount that would reduce the turbidity produced by 0.25 mg. of chondroitin sulfate under the specified conditions to that produced by 0.125 mg.

RESULTS

Mixed cultures: Chondrosulfatase was present in all of the mixed cultures of the subgingival accumulations from 10 subjects who presented a clinical picture of chronic marginal gingivitis and in 8 of the cultures from 11 subjects whose gingivae were clinically normal. Readily cultivable microorganisms that produced this enzyme were therefore almost universally present in the gingival crevices and pockets sampled. Only the undiluted culture supernates were tested. Uniformly, they hydrolyzed all of the substrate under the conditions of the assay method.

Pure cultures: Prior to the search for microorganisms responsible for the production of chondrosulfatase in the above mixed cultures, we tested for this enzyme in beef-extract broth cultures of combinations of fusiform bacilli and other gingival microorganisms that had been shown previously to produce phenolsulfatase.[2] These tests were of importance because phenolsulfatase may contribute to the breakdown of chondroitin sulfate by removal of the sulfate group,[15] and organisms producing it might also attack the polysaccharide chain of chondroitin sulfate. However, none of the combinations produced chondrosulfatase (Table I), although they formed phenolsulfatase in control cultures grown simultaneously.

TABLE I
PRODUCTION OF CHONDROSULFATASE BY COMBINATIONS OF SINGLE COLONY STRAINS OF GINGIVAL BACTERIA

TYPES	NO. POSITIVE / NO. TESTED
Staphylococcus + fusiforms*	0/7
Streptococcus + fusiforms*	0/7
Viridans streptococci + fusiforms*	0/5
Diphtheroid (aerobic) + fusiforms*	0/5
Gram-negative cocci + fusiforms*	0/9
Staphylococcus + diphtheroid (aerobic)	0/18
Diphtheroid (micro-aerophilic) + staphylococcus	41/41
Diphtheroid (micro-aerophilic) + diphtheroid (aerobic)	3/3

*Combinations that produced phenolsulfatase in a previous study.[2]

We also tested many of the strains of viridans streptococci that had been examined previously[1] for hyaluronidase and beta-glucuronidase production (Table II). Of the 33 single colony strains tested, 11 produced hyaluronidase and 9 produced beta-glucuronidase. Of the 28 pooled strains, 13 made hyaluronidase and 8 made beta-glucuronidase. None of these 61 cultures formed chondrosulfatase.

TABLE II
PRODUCTION OF CHONDROSULFATASE BY SINGLE COLONY STRAINS OF GINGIVAL BACTERIA

TYPES	NO. POSITIVE / NO. TESTED	
	AEROBIC	ANAEROBIC
Viridans streptococci*	0/33	0/12
Viridans streptococci (pooled strains)*	0/28	
Fusiforms		0/7
Staphylococci	0/84	0/8
Beta streptococci	0/22	0/11
Gram-negative cocci	0/45	0/10
Diphtheroids (aerobic)	0/54	0/16
Diphtheroids (micro-aerophilic)	15/17	16/16

*Strains used previously in a study of hyaluronidase and beta-glucuronidase production.[1]

The search for microorganisms that produced chondrosulfatase was concentrated on the mixed broth cultures from 4 of the subjects having chronic marginal gingivitis. When these cultures were streaked out on solid media, it was easy to demonstrate that the chondrosulfatase producer was present where the resultant growth was heavy and mixed, but that it was generally absent where the colonies were well separated. This observation indicated a symbiotic relationship, which was subsequently identified. Chondrosulfatase was not produced by pure cultures of any of the more evident bacteria, such as staphylococci, beta streptococci, micro-aerophilic gram-negative cocci (probably of the genus, *Veillonella*), nor by a typical diphtheroid bacillus designated for convenience as "aerobic" (Table II). Likewise, several combinations of these organisms failed to make the enzyme (Table I). Owing to its satellitory habit of growth, the responsible microorganism was eventually shown to be present in mixed colonies consisting of staphylococci and small gram-positive bacilli.

Isolation and characterization of the responsible microorganism: Bordet-Gengou sheep blood agar was found to be the most favorable of the media tested for the isolation of the responsible organism. The mixed colonies of staphylococci and gram-positive bacilli were streaked out on this medium and incubated anaerobically under a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide at 37° C. After 72 hours, tiny light yellow secondary colonies consisting of the gram-positive bacilli appeared on the top of the white colonies of staphylococci. On continued incubation, these papillae increased in size and changed color progressively to brownish yellow and light brown. After from 72 to 96 hours of incubation they could be picked by a needle with minimal admixture of staphylococci and grown as isolated pure colonies on the Bordet-Gengou medium, incubated anaerobically as before.

Colonies were not readily evident until after 72 hours, and even after 96 hours their diameter did not exceed 0.2 mm. They were nearly hemispherical and remained typically shiny, moist, and mucoid in appearance throughout the entire period. Like the symbiotic colonies with staphylococci, the isolated colonies progressed with age from light yellow to brownish yellow to light brown. Where the colonies were crowded, the area was surrounded by a gray zone with a black border. On ordinary sheep blood agar, the colonies were of the same size, but nonpigmented, and there was no discoloration of the medium.

Microscopically, the organisms grown on the Bordet-Gengou medium were small gram-positive bacilli, 0.5 μ by 1 to 2 μ (Fig. 1). Polar staining was so pronounced that differentiation from streptococci was often difficult. When grown in liquid media, however, a typical diphtheroid arrangement became evident (Fig. 2). Staining with toluidine blue did not reveal definite metachromatic granules. The cellular morphology was unchanged when the bacilli were grown on Loeffler's medium. There was only a slight tendency to pleomorphism, even after incubation for 8 days.

The organism grew much more readily in liquid media. In the beef-extract broth, growth was visible macroscopically within 24 hours anaerobically at 37° C. In contrast to the solid media, this broth supported the growth of the bacillus "aerobically" provided that a large inoculum was used. The organism, therefore, is "micro-aerophilic" and not obligatorily anaerobic. In thioglycollate broth without dextrose (Difco), growth was of the same magnitude as in the beef-extract broth anaerobically. The terminal pH in this broth was 7.0. In thioglycollate broth with dextrose (Difco) the growth was extraordinarily dense within 24 hours beneath the surface. Acid was formed, but not gas, and the terminal pH (after 72 hours) was 5.1.

Because of the present state of bacterial taxonomy, the specific identification of a "diphtheroid" is problematic. The microorganism under discussion does not conform to any of the species of *Corynebacterium* recognized by Bergey's Manual. It has, therefore, been described in detail as a guide to its recognition by other investigators. Two criteria seem to exclude it from *Actinomyces* and *Lactobacillus*, the two genera most likely to cause confusion: its morphology and the acidity produced from dextrose. Even after 8 days in thioglycollate broth (with and without dextrose) the cells looked very little different from those of a 24-hour culture and there was no evidence of the branching filaments characteristic of an actinomycete. Meanwhile, in thioglycollate broth with dextrose, the pH fell to a low of 5.1 within 3 days and rose slightly to 5.3 by the end of 7 days of incubation at 37° C. A lactobacillus would have been expected to produce a considerably lower pH and there would probably have been no reversal.

On the basis of the foregoing information, the microorganism in question is characterized simply as a micro-aerophilic diphtheroid bacillus.

Chondrosulfatase production: Under anaerobic conditions, growth of the above characterized diphtheroid bacillus in the beef-extract medium was

sufficient within 48 hours at 37° C. to demonstrate the production of chondrosulfatase by every one of the 16 single-colony strains tested (Table II), which were isolated from 4 subjects. When a heavy inoculum was used, similar results were obtained after aerobic incubation. In 2 cases out of 17,

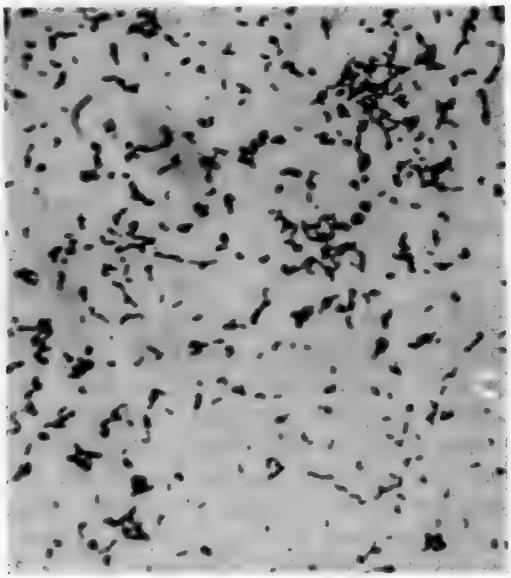

Fig. 1.—Chondrosulfatase-producing diphtheroid bacillus. Four-day anaerobic culture on Bordet-Gengou agar. (Crystal violet stain; ×980.)

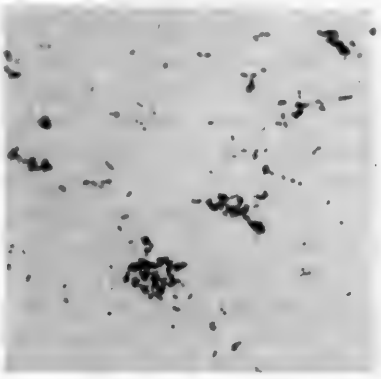

Fig. 2.—Chondrosulfatase-producing diphtheroid bacillus. Two-day anaerobic culture in beef-extract broth. (Crystal violet stain; ×980.)

however, the growth was sparse and no chondrosulfatase was formed. The potency of the enzyme in the broth cultures varied from 3 to 5 turbidity reducing units per milliliter. When single colonies of *Staphylococcus albus* isolated from human gingival crevices were added to the broth along with

the diphtheroid and the culture was incubated aerobically, both microorganisms grew abundantly and chondrosulfatase was present within 24 hours (Table I). A similar effect was produced by addition of aerobic diphtheroids isolated from human gingival crevices. Since the micro-aerophilic diphtheroid grows perfectly well in this broth and produces the enzyme under anaerobic conditions, it is not likely that the staphylococci and the aerobic diphtheroids were supplying any growth factors. Rather, it is suggested that these symbiotic relationships depend upon the nonspecific reduction of the oxidation-reduction potential by the aerobic microorganisms to a level suitable for the growth of the micro-aerophilic diphtheroid.

Although dextrose added to brain-heart infusion broth is favorable to the production of hyaluronidase by various microorganisms, chondrosulfatase activity was absent from any broth culture tested that contained dextrose. Thioglycollate broth with dextrose supported the growth of the chondrosulfatase-producing diphtheroid far better than any other medium tested, but the cultures did not exhibit chondrosulfatase activity. Thioglycollate broth without dextrose supported adequate, though less abundant growth, but again no enzyme could be demonstrated. Thioglycollate broth, therefore, contains substances other than dextrose that inhibit the formation or the action of chondrosulfatase.

Under the conditions of the present experiments, chondrosulfatase was produced constitutively, that is, even when the bacillus was grown in the absence of the specific substrate. It evidently passed readily from the cells into the medium. The amount produced, however, was approximately doubled when the organism was grown in the beef-extract broth with the addition of 0.025 mg. of chondroitin sulfate per milliliter.

Specificity: The significance of the kind of results reported here is critically dependent upon the purity of the substrates used. In the case of chondroitin sulfate, the most important impurity to be expected is probably hyaluronic acid. However, hyaluronidase produced by viridans streptococci isolated from human gingival crevices had no effect on the chondroitin sulfate preparation employed in the present experiments (Table II). It follows that these streptococci did not produce chondrosulfatase. Cultures of the micro-aerophilic diphtheroid in brain-heart infusion broth with added dextrose, as well as in beef-extract broth, were tested for hyaluronidase by the method of Tolksdorf and associates.[14] The results indicated an average of 0.25 turbidity reduction units of enzyme per milliliter of culture. Since the cultures in the brain-heart infusion broth did not contain chondrosulfatase, it appeared that the micro-aerophilic diphtheroid produced small amounts of hyaluronidase. The action of the cultures in beef-extract broth, however, may have been due to the combined action of hyaluronidase and chondrosulfatase, for hyaluronic acid prepared from human umbilical cords regularly contains chondroitin sulfate as an impurity.

DISCUSSION

It has been demonstrated that microorganisms isolated from human gingival crevices and pockets produce chondrosulfatase, hyaluronidase,[1] beta-glucuronidase[1] and phenolsulfatase.[2] These findings indicate the possibility that an important function of bacteria in the development of chronic marginal gingivitis and periodontal disease may be the disruption of the mucopolysaccharides of gingival tissues. The hydrolysis of the intercellular substances might aggravate the tissue sufficiently to account for a part of the inflammatory reaction. Also this hydrolysis would be expected to afford pathways of increased tissue permeability for the diffusion of toxic substances known to be present in gingival crevices and pockets.

Evidence was presented previously that hyaluronidase and sulfatase remove intercellular substances from gingival tissue.[16] The enzymes, however, were in the form of testicular extracts, which might have a different action than analogous bacterial enzymes. Also, testicular extracts contain a number of enzymes in addition to hyaluronidase, beta-glucuronidase, and sulfatase.

The fact that microorganisms producing these enzymes are found frequently in the subgingival accumulations of individuals with clinically healthy gingivae does not contradict the view formulated above. In the first place, the responsible bacteria might be present in such small numbers at a particular juncture that they would produce nothing more than the microscopically detectable degree of gingivitis apparently universally present in man, as judged from observations on histologic sections of clinically normal gingivae. Secondly, it is a general principle that any microbial potentiality may fail to manifest itself unless the milieu be favorable. In the present experiments, for example, chondrosulfatase was not formed whenever dextrose was present in the culture medium, and was not formed in thioglycollate broth even in the absence of dextrose. On the contrary, it is probably significant that the various microorganisms tested produced more of the enzymes in question when they grew in the presence of the specific substrate, as they presumably do in their natural habitat. We do not know as yet, of course, what may be the conditions in the gingival crevice that control the growth of the resident bacteria and the nature of their products. Finally, one has to consider that any microbial attack is conditioned by the status of the host tissues, locally or generally.

It is not implied that the micro-aerophilic diphtheroid bacillus described here is the only oral microorganism that produces chondrosulfatase, although no other was evident in the mixed cultures investigated in the manner described.

SUMMARY

Chondrosulfatase was produced by all of 10 mixed bacterial cultures obtained from the gingival pockets of 10 individuals exhibiting chronic marginal gingivitis and by 8 of 11 such cultures obtained from the gingival crevices of 11 subjects with clinically normal gingivae. A micro-aerophilic diphtheroid bacillus was the only organism isolated from these cultures that produced

chondrosulfatase. The enzyme was not produced by alpha and beta streptococci, staphylococci, gram-negative micro-aerophilic cocci, aerobic diphtheroid bacilli, and fusiform bacilli isolated from human gingival pockets, nor by various combinations of these bacteria.

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A METHOD FOR STUDYING "THE EPITHELIAL ATTACHMENT"

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INTRODUCTION

BLACK[1] wrote in 1915 that ". . . a thin blade may readily be passed between the gingiva and the enamel, raising the tissue and exposing the subgingival space between it and the enamel. This tissue has no attachment to the tooth until the gingival line on the tooth is reached." This clinical observation of Black's on the relation between the gingiva and the enamel has disappeared from our textbooks since Gottlieb[2] presented, in 1921, his concept of "the epithelial attachment" based upon microscopic observations. Bodecker and Lefkowitz[3] (1935) expressed the "enlightened clinicians' dilemma": "The demonstration of the epithelial attachment to the dental enamel in man is difficult clinically, because an instrument usually can be passed readily along a tooth to the cementoenamel border. This gives an impression that the epithelium only overlaps the enamel. In truth the instrument severs the attachment without any resistance or hemorrhage." Waerhaug[4] (1952) questioned "the epithelial attachment concept" in a monograph reporting his own experimental and histologic work.

For review of the literature pertaining to the epithelial attachment and the gingival sulcus the reader is referred to papers by Orban and Mueller,[5] Waerhaug,[4] and Baume.[6] Ramfjord and Kiester[7] (1954) studied the gingival sulcus after scaling the teeth. In this study, histologic observations followed a clinical procedure of entering the gingival sulcus with scalers. In each of the cases reported, the bottom of the clinical pocket coincided with the attachment of connective tissue. In no case was the epithelial attachment strong enough to withstand the scaling operation with conventional instruments. Such studies as this and the ones reported by Waerhaug are responsible for a re-evaluation of the significance of the epithelial attachment on enamel and cementum in the practice of dentistry—more specifically with the re-evaluation of such questions as: Should the epithelial attachment be removed during curettage or gingivectomy in the treatment of periodontal disease? How far should the margin of restorations be placed underneath the gingiva in relation to the epithelial attachment? How strong is the epithelial attachment? What happens if it is detached?—and many others.

The object of this report is to present a simple reproducible method by which clinical measurements and observations can be correlated with histo-

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logic observations of the epithelial attachments. Such a method is necessary because clinically it is impossible to observe or test the epithelial attachment. Yet the concept of this attachment has dominated the thinking of our clinicians.

EXPERIMENTAL PROCEDURE

The principle of the method consists of the insertion of a very fine strip of film between the gingiva and the tooth surface. The film material must be flexible and yet tough enough to enter into the gingival sulcus. It must withstand ordinary laboratory procedures for the fixation, sectioning, and staining of histologic specimens.

In the present study a cellulose acetate film,* 0.1 mm. thick, was used.† It can easily be cut into strips of any desired width and length. It is impossible to exert more than a very slight amount of force when small strips of this material are pushed into the gingival sulcus. There are too many variables involved to measure exactly the amount of force needed for the insertion—such as the width and length of the strip, and the friction between the material and the tooth on one side and the soft tissue on the other. Therefore, unscientific as it may seem, the best expression of the maximum amount of force that can be exerted with this material in the process of insertion is "slight."

If it is desired to maintain the cellulose acetate film for any length of time within the gingival pocket, a cavity must be prepared into the tooth coronally to the gingival margin. The end of the film can then be folded into the cavity and held there with a self-curing resin-filling material. After the specimen is decalcified, it is placed in chloroform for 24 hours to dissolve the film.

The thickness of 0.1 mm. was chosen arbitrarily. This material is available in thicknesses from 0.001 to 0.02 inch.‡ It is possible to measure the length of the film strip when it is inserted into the sulcus or pocket. It can be measured again in the microscopic section, and its relation to the histologic structures can be observed. Great care is necessary during the removal of the specimen for fixation in order not to destroy the film-tissue-tooth relationship.

DISCUSSION OF RESULTS

Figs. 1, 2, and 3 are photomicrographs of a histologic section from an upper incisor of a young dog, showing a cellulose acetate strip in place and its relation to the tooth and the gingival tissues. The film was held in this position for 8 weeks after having been inserted into the gingival sulcus. The end of the film lies near the cementoenamel junction (*CE*); the distance from

*Kodapack IV, Eastman Chemical Products, Inc., Kingpoint, Tenn.

†This material is found in dental offices under the trade name of Resin Slab, The L. D. Caulk Company, Milford, Del.

‡The new polyester films manufactured by duPont and marketed under the trade name of Mylar may be even more suitable. They are available in thicknesses from 0.0005 inch.

Fig. 1.—Photomicrograph of cellulose acetate film strip, 8 weeks after insertion (magnification ×33). *A*, Bottom of "gingival sulcus; *CE*, cementoenamel junction.

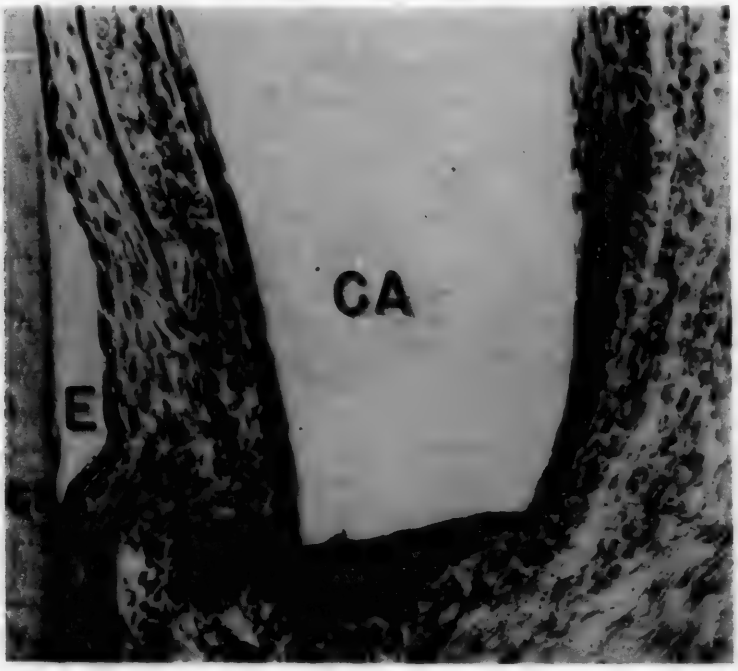

Fig. 2.—Higher magnification from Fig. 1 of lower end of cellulose acetate film in relation to cementoenamel junction (*CE*) and gingival tissues (magnification ×325). *E*, Enamel; *CA*, cellulose acetate film.

the crest of the gingiva to the bottom of the sulcus measures 2.4 mm. Between the lower end of the film and the tooth surface is a space of 0.1 mm. filled with soft tissue.

Several incidental observations can be made on this specimen. The microscopic appearance of the epithelium-film-junction is identical with that of an epithelium-enamel-junction (Fig. 2). A pink-staining cuticle can be recognized on both the enamel (*E*) and all 3 film surfaces in Fig. 2. If the viewer did not know that this is a cellulose acetate film-epithelium junction, he would interpret it according to current concepts, as an enamel-epithelium junction with the bottom of the sulcus at *A* in Figs. 1 and 3. Below this point the epithelium is attached according to the pictures in textbooks of dental histology (Figs. 183 and 184, Orban[9]).

Fig. 3.—Higher magnification from Fig. 1 of cellulose acetate film-epithelial junction (magnification ×330). *A*, Bottom of "gingival sulcus."

The epithelium-cellulose acetate junction is also comparable to the epithelium-acrylic junction described by Waerhaug.[8] In control teeth from the same animal, where the acetate film was not inserted in the gingival sulcus, the most apical epithelial cells are at or near the cementoenamel junction. In Figs. 1 and 2 the lowest epithelial cell is located 0.2 mm. below the cementoenamel junction.

The use of this described method should clarify some aspects of the clinical significance of the "epithelial attachment." Studies toward that end are now in progress.

CONCLUSIONS

1. The method described permits a correlation of clinical observations of sulcus and pocket depth with histologic findings.

2. Eight weeks after insertion of a strip of cellulose acetate film into the normal gingival sulcus of a dog's incisor, an epithelium-cellulose acetate junction had an appearance similar to that of "enamel epithelial attachment."

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ENAMEL CUTICLE

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EARLIER INVESTIGATIONS

THE cuticular substance which covers the enamel is defined by Kronfeld[8]:

> The primary cuticle, the last product of the ameloblasts, is formed on the enamel surface before the ameloblasts degenerate and disappear. It is about 1 micron thick and usually becomes calcified. . . . The secondary cuticle is a horny structure formed by the squamous cells of the epithelial attachment. . . . It appears bright pink when stained in eosin, and is about 7 micra thick.

Nasmyth[11] seems to have been the first to call attention to a "persistent dental capsule" on the surface of the enamel. The capsule could be floated off when the tooth was submerged in acid and is described as "fibrous externally and having on its internal surface the peculiar reticulated appearance common to the tooth." Nasmyth's experiment has been repeated by many others and, until 1921, 3 main theories concerning the formation and the structure of the enamel cuticle were at hand.

1. According to the first of these theories which was based on Nasmyth's work, the cuticle was supposed to be a thin layer of cementum formed by the connective tissue of the dental sac.

2. Another group, headed by Kolliker and von Ebner,[3] believed that the cuticle was formed by the inner enamel epithelium when the enamel formation was finished. It was emphasized that the cuticle was calcified and that it was part of the enamel.

3. A third theory maintained that the cuticle was formed by the outer enamel epithelium after the degeneration of the ameloblasts, the inner enamel epithelium, and the stratum intermedium. This theory stressed that the cuticle is hornified and not calcified. This line of thought was formulated by Waldeyer.[17]

Gottlieb[6, 7] seems to have been the first to subject the enamel cuticle to a thorough histologic examination. His theory, which is prevalent in modern literature, is a compromise between that of Kolliker and von Ebner and that of Waldeyer. Kronfeld's definition is based on this theory. Unfortunately Gottlieb gave no clue to distinguishing between two cuticles when they occur together. Usually the cuticle is described as primary when it is very thin, and as secondary when it is thicker and when a definite horn stain can be detected.

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Recent experimental investigations on dogs have shown that a membrane corresponding to the description given of the "primary cuticle" could be observed on the surface of acrylic crowns[16] and cellulose acetate films[18] which had been in contact with the gingiva for some weeks. In certain areas this membrane was thicker and took the horn stain in a manner corresponding to the definition given of the "secondary cuticle." Observations[15] under the stereomicroscope of the surface of unfixed extracted human teeth, treated with horn stain, revealed a thin membrane which took the typical horn stain. This membrane, which at a histologic control proved identical with the so-called "secondary cuticle," had a puttylike consistency. Since a similar "secondary cuticle" could also be found on the surface of artificial crowns that had been in contact with the gingiva for some time, this cuticle does not occur exclusively on the natural tooth.

The nature of the enamel cuticle has also been discussed by many others.[1, 2, 4, 5, 9, 10, 12, 14]

STATEMENT OF PROBLEM

The finding of a "primary cuticle" on the surface of acrylic crowns and cellulose acetate films indicates that the extremely thin membrane which is usually found between the epithelium and the enamel need not necessarily be a fixed anatomic entity formed by the ameloblasts as their last product.[6, 7] The object of this investigation is to find out whether a membrane similar to the "primary cuticle" will reappear if the surface of the enamel is completely ground away below the gingival margin. The result of such an operation may throw some light on the consistency and the origin of the enamel cuticle.

MATERIAL AND METHOD

Twenty teeth in 3 young monkeys and 15 teeth in 5 young dogs were used for the experiment. On 13 of these teeth (6 in monkeys and 7 in dogs) the experiment was carried out both on the buccal and lingual sides. On the rest of the teeth (22) only one surface was used, the other side being kept as a control surface. This gives a total of 48 tooth surfaces used. A very thin diamond point was inserted into the pocket until it met definite resistance. The surface of the enamel was then ground away in the entire area between the gingival margin and the line to which the diamond point reached. A nick was made in the tooth surface indicating the depth to which the grinding had been carried out. The diamond point, which was new and sharp, left a rough surface which was not polished. During the grinding the soft tissue was not kept aside, but was left in contact with the rotating diamond point. For that reason, some of the soft tissue was also removed. The gingiva was then allowed to heal for a period extending from 23 to 216 days. Two of the dogs contracted distemper shortly after the operation and had to be sacrificed after 23 and 37 days, respectively, at which time they were in extremely poor condition. The 4 monkeys had their teeth brushed on the left side during the observation period, the right side being kept as control in order to see whether oral hygiene in any way influenced the healing. The animals were decapitated after having been anesthetized with Nembutal. The jaws were taken out very carefully and fixed in 10 per cent

formalin. They were decalcified in nitric acid and embedded, some in paraffin and some in celloidin. Serial sections were obtained from the entire buccal and lingual surfaces, and about every tenth of these was examined. The sections were stained with hematoxylin and eosin and in some cases with a bacterial stain. The paraffin sections were 6 to 9 microns thick and the celloidin sections 12 to 14 microns.

OBSERVATIONS

Six photomicrographs showing typical pictures from this experimental series will be shown. The nick made by the diamond point is easily seen in the dentin below the cementoenamel junction (Figs. 1 to 3). In Figs. 4 and 5 the diamond point has not reached the cementoenamel junction, but a distinct step in the surface of the epithelium indicates the depth to which the grinding has been carried out *(JUNCT)*. The epithelium bordering upon the ground enamel surface above the junction is uneven as compared with that bordering upon the untouched surface below it. In all the illustrations a distinct cuticle, *CU*, is seen between the ground enamel surface and the epithelium. The regeneration of the removed epithelium is complete, and healthy normal epithelial cells join closely to the cuticle. In some cases a number of polymorphonuclear leukocytes are seen between the epithelial cells (Figs. 1 and 3). In other cases this cellular infiltration is not present in all areas (Figs. 2, 4, 5, and 6). Subgingival bacterial plaque was found between the epithelium and the tooth in a number of cases (Figs. 2 and 4). Between the plaque and the epithelium a number of polymorphonuclear leukocytes were usually found (Fig. 4, *PL*), and corresponding to the plaque a more or less pronounced infiltration in the connective tissue was present. Healing seemed to take place in the same way both in the dogs and in the monkeys.

On all the 35 teeth on which this operation was carried out there was found a cuticle which in appearance corresponded to that found on the control surfaces. The only difference was that the control cuticles usually formed an absolutely straight line, while the cuticles found on the ground enamel surfaces usually were curved, fitting into the roughnesses in the enamel. The thickness of the cuticle as measured in the sections was generally very close to 1 micron (Figs. 2 to 6). Occasionally it could be 2 microns (Fig. 1). No consistent difference could be observed between the thickness of the cuticle on the experimental surfaces and that on the control surfaces.

A general observation in this material was that the cuticle came out more clearly in the sections taken from the central part of the tooth (Fig. 7, *A*). In the sections from the periphery there was often no cuticle to be seen (Fig. 7, *B*).

DISCUSSION

In all the cases where the enamel surface had been ground away a more or less complete healing appeared within 23 days or more. A new epithelial cuff was formed, and in some cases this cuff joined tightly to the tooth all the way to the gingival margin. In some cases a comparatively great number of polymorphonuclear leukocytes were seen between the epithelial cells, as in Figs. 1 and

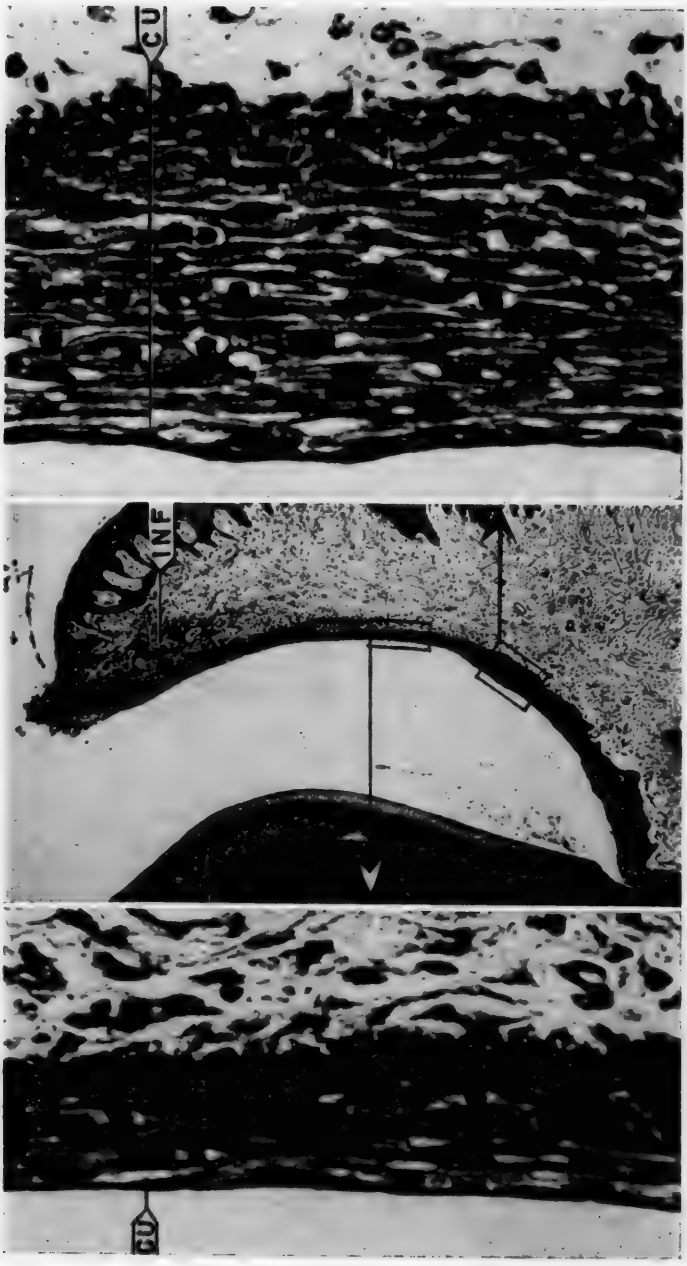


Fig. 1.—Paraffin section, Dog 10. Observation period 37 days. Distemper during entire experiment. Enamel surface ground away from gingival margin to nick in cementum below cementoenamel junction. In higher magnifications to right and left, a distinct cuticle, *CU*, is seen on epithelial surface.

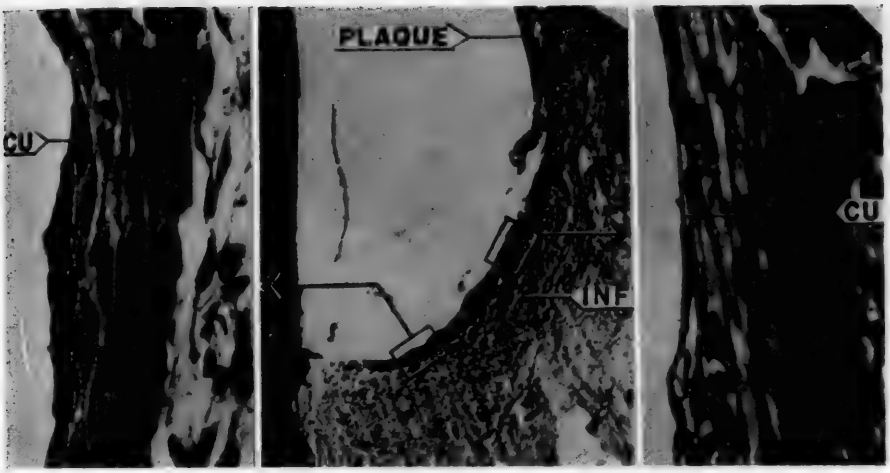


Fig. 2.—Paraffin section, Dog 9. Observation period 23 days. Distemper during entire experiment. Technic as in Fig. 1. Plaque formed below gingival margin. Infiltration in connective tissue. Higher magnifications show formation of cuticle, *CU*, on rough enamel surface. To the left, normal epithelial cells. To the right, epithelial cells degenerated.

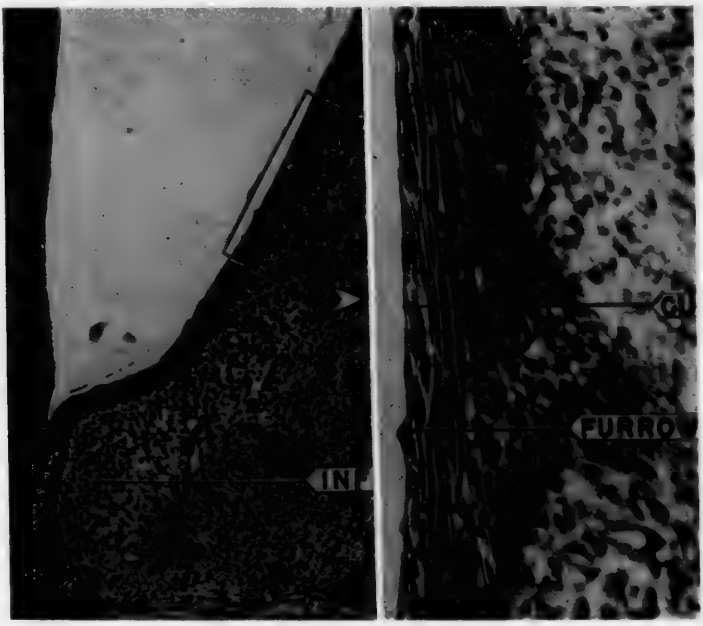
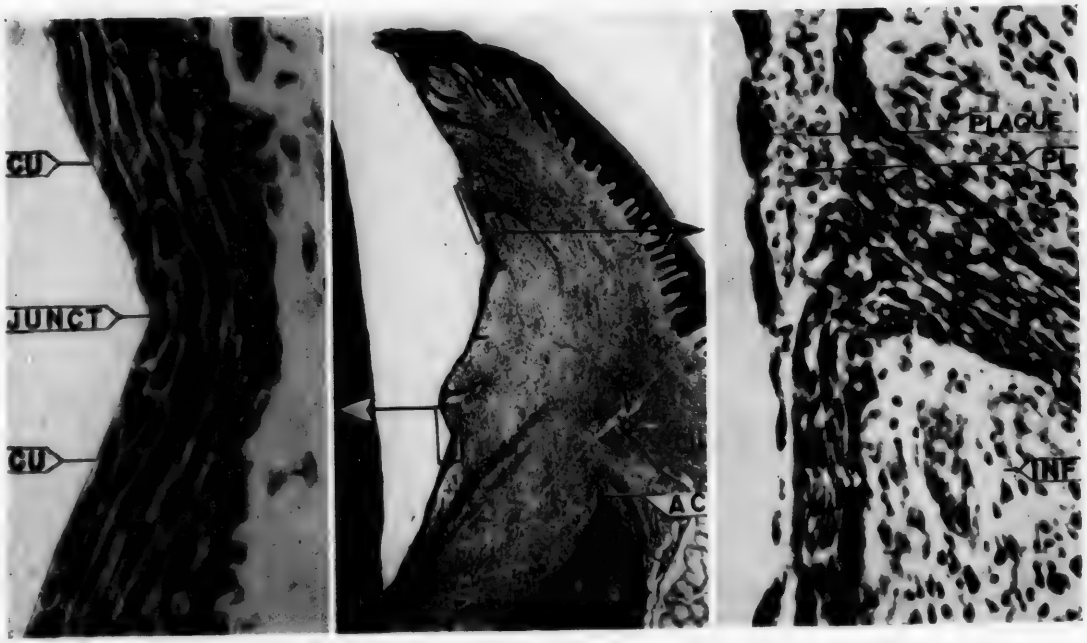


Fig. 3.—Celloidin section, Monkey 3. Observation period 40 days. Technic as in Fig. 1. Normal epithelial cells joining cuticle, *CU*, on ground enamel surface. Some polymorphonuclear leukocytes between epithelial cells. Infiltration in connective tissue.



A. *B.* *C.*

Fig. 4.—Celloidin section, Monkey 7. Observation period 23 days. Surface of enamel ground away. Junction between ground and intact surface seen in higher magnification to the left. Cuticle on unground enamel continues onto rough surface of ground enamel. In higher magnification to the right, subgingival bacterial plaque and polymorphonuclear leukocytes, *PL*.

Fig. 5.—Celloidin section, Monkey 7. Observation period 23 days. Surface of enamel ground away. Junction between ground and intact surface seen in higher magnification to the right. Close adaptation of epithelium against enamel to gingival margin. Cuticle on unground surface below junction somewhat thinner than cuticle on rough ground surface above junction.

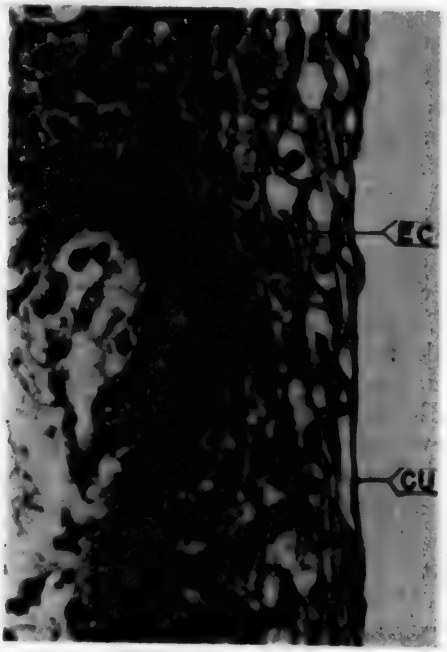


Fig. 6.—Paraffin section, Monkey 1. Observation period 135 days. Magnification ×500. Enamel surface ground away below gingival margin. Cuticle, *CU*, fitting into rough surface of enamel. Cell fallen out making the cuticle more visible. *EC*, epithelial cuff.

3. In other cases many areas of the epithelial cuff were normal (Figs. 2, 4, 5, and 6). This confirms earlier observations[15] that epithelium will join closely to any surface as long as this surface does not cause irritation.

It was also a consistent observation that a cuticle comparable to what has been described as the "primary cuticle" could be found in the areas where the enamel surface definitely had been ground away. The cuticle was in most cases about 1 micron thick, as is said to be one of the requirements for identification. Figs. 4 and 5 were selected because both the original cuticle and the one formed on the ground surface appear in the same photomicrograph. The two cuticles should, consequently, be absolutely comparable as far as morphology is concerned. In Fig. 4 the original cuticle passes without break in continuity into the cuticle on the ground surface, the latter being a little thinner and less distinct. In Fig. 5 the cuticle on the ground surface is the thickest and the most distinct one. The fact that the two cuticles have the same appearance in the histologic section is no proof that they are formed by the same material and in the same way, but that possibility must be seriously considered. In a decalcified stained section it is impossible to decide whether or not a cuticle of that thickness is calcified, but calcification is rather unlikely as similar cuticles could be observed on the surface of acrylic crowns[16] and cellulose acetate films[18] which had been in contact with the gingiva for some weeks. There is, consequently, reason to believe that the 1 micron thick membrane usually found between the enamel and the epithelium ("primary cuticle" according to the definition given earlier) is organic and of epithelial origin, formed by the epithelial cells which happen to lay in contact with the enamel. Whether these cells are ameloblasts or squamous cells from an epithelial cuff does not seem to make any difference. In Fig. 6 a cell from the epithelial cuff has fallen out, thus making the cuticle more visible. This picture corresponds in most details with the illustration of the "primary cuticle" in Orban's *Histology and Embryology*, Fig. 45.[13]

The cuticle seen in Fig. 1 is somewhat thicker than those in the other illustrations, and a slight pink color could be seen which corresponded to the definition of the "secondary cuticle." There is no evidence, however, that there should be two cuticles beside each other. Thus, there may be no qualitative difference between the "primary" and the "secondary" cuticle. Deposited in thicker layers the membrane may take more stain and appear pink when stained with eosin. In thin layers the color may not come out so clearly. Optical phenomena may also play a role. Until further evidence has been provided to the proof that there really are two cuticles of different consistency and of different origin, it may be wise to use the more unspecific term, enamel cuticle.

It was also apparent that the angle of the epithelial surface to the direction of the sectioning played a role, as the best pictures of the cuticle generally were obtained in sections from the middle of the block where the epithelium was sectioned at a right angle (Figs. 7 and 8). When looking at Fig. 7, *B*, it is easy to understand that the extremely thin cuticle may fail to show up if it is viewed at too great an angle to the optical axis of the microscope. This may explain why some research workers have failed to find a cuticle in certain cases. The

unsharp appearance of the cuticle below *CU* in Fig. 6 is due to a minor inclination of the cuticle. The microscopic objective has a depth of focus of approximately 2 to 3 microns. This means that areas immediately above and below the focus will be more or less unsharp, according to the distance from the focus. If the cuticle is oblique in relation to the optical axis, the unsharp areas will be projected besides the sharp area.

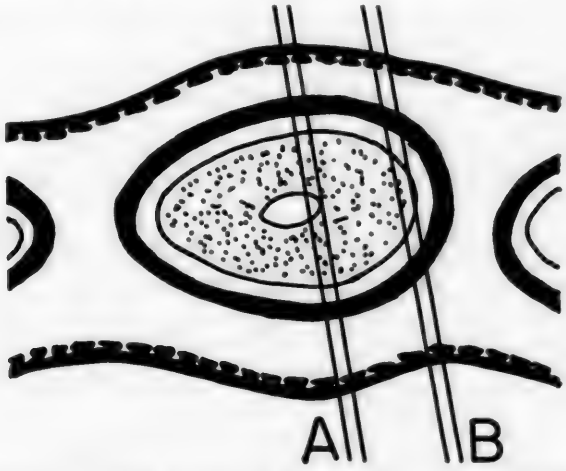


Fig. 7.—Schematic drawing horizontally through jaw in area between gingival margin and cementoenamel junction. Buccolingual section from central part, *A*, cut perpendicularly to enamel surface. Section from periphery, *B*, cut at angle of about 45 degrees to enamel surface.

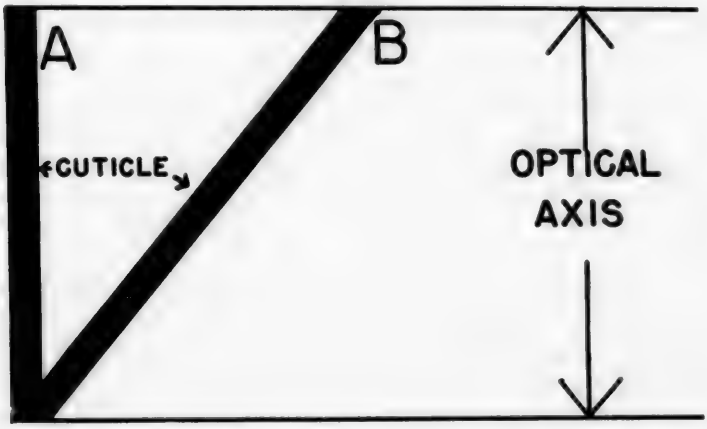


Fig. 8.—Schematic drawing illustrating 12 microns thick histologic section of 1 micron thick enamel cuticle. In *A*, where section is taken from central part of tooth corresponding to *A* in Fig. 7, cuticle is viewed in microscope parallel to optical axis. In *B*, where section is taken from periphery corresponding to *B* in Fig. 7, cuticle is viewed at angle of 45 degrees.

For the optical microscopic sections, 5 to 15 microns thick are used, and sections of that thickness are not suited for studying fine details in membranes of 1 micron or less. This seems to limit the possibilities of the optical microscope. The electron microscope has in this case an enormous advantage, as sections of

0.1 micron are used. Such a section would seem as thick as the line at the bottom in Fig. 8 when compared with a 12 micron thick celloidin section, and distortion would be of no practical influence.

CONCLUSIONS

1. The membrane about 1 micron thick found on the surface of the epithelial cuff after decalcification of the enamel, usually called the "primary enamel cuticle," may not represent an anatomic entity formed by the ameloblasts as their last product.

2. The "primary cuticle" may be formed by the epithelial cuff and may not be calcified.

3. The difference between the "primary" and the "secondary" cuticles may lie in their thickness and not in their quality. In fact, there may be only one cuticle.

4. A cuticle which is present on the surface of the epithelial cuff may be unobservable in the microscope if it is not sectioned at a comparatively right angle.

SUMMARY

On 48 tooth surfaces in 3 monkeys and 5 dogs the enamel surface was ground away below the gingival margin. The animals were sacrificed after 23 to 216 days. The histologic sections showed reappearance of a thin cuticle corresponding to the description usually given of the "primary enamel cuticle."

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EFFECT OF ROUGH SURFACES UPON GINGIVAL TISSUE

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EARLIER INVESTIGATIONS

IT IS generally agreed that unpolished restorations situated below the gingival margin will cause a more severe irritation than well-polished ones. Experimental histologic investigations[2] have shown that the epithelium under favorable conditions can join the surface of acrylic crowns in the same way as a normal epithelial cuff[1] (epithelial attachment) joins the tooth. Signs of irritation were found mainly next to the junction between the tooth and the artificial crown, or in places where large remnants of cement had been left in contact with the soft tissue. From this the conclusion was drawn that zinc-oxyphosphate cement acts as an irritant upon the gingiva while a polished acrylic surface does not.

It also has been generally accepted that subgingival calculus acts as an irritant upon the gingival tissue on account of its roughness. Experimental bacteriologic investigations[3] have shown that pockets with subgingival calculus always contain bacteria, while pockets without subgingival calculus usually are sterile. Even pockets around well-fitting artificial crowns can, under favorable conditions, be sterile. From this the conclusion was drawn that the bacteria are the main source of irritation in calculus.

Where cement is concerned it is impossible to decide whether the irritation is caused by chemical agents in the cement itself or by its rough surface injuring the epithelium mechanically. Where calculus is concerned it is in the same way impossible to determine whether the irritation is caused by the bacteria alone, or whether the uneven surface of the calculus to a certain degree plays a role.

The following work was carried out to determine whether or not a decidedly rough surface below the gingival margin will prevent healing and act as a permanent irritant.

MATERIAL AND METHOD

The material is the same as that previously described.[4] The subgingival enamel surface was ground away with a new and very sharp diamond point. This produces a rough surface, as the grains are very sharp, making deep furrows and high ridges. The appearance of an enamel surface ground with the same diamond point is seen in Fig. 1, photographed in the metalloscope.

OBSERVATIONS

For the description of the sections the reader is referred to the earlier publication.[4] The inflammation in the connective tissue as a rule was more pro-

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nounced on the experimental side than on the control side. This was especially true in the shorter observation periods. In 14 pockets in the monkeys and in 8 pockets in the dogs a certain amount of subgingival calculus or plaque similar to that described in Figs. 2 and 4[4] was seen. On the control side or control teeth subgingival calculus was found only in one case. In the 3 monkeys in which toothbrushing was carried out during the observation period, there was no obvious difference between the brushed and the unbrushed side.

In the 2 dogs which suffered from distemper the healing seemed to progress in the same manner as in the healthy animals.

DISCUSSION

It is apparent from Fig. 1 that the diamond point produced a very rough surface, most likely rougher than would be expected on a restoration or on subgingival calculus.

Fig. 1.—Tooth surface ground with coarse diamond point (magnification ×70).

In all the teeth examined in this experiment there was a complete readaptation of a new healthy epithelial cuff to the ground surface in most areas. This means that this artificially produced rough surface did not in itself exert any irritating effect on the epithelial cells. In all the illustrations it may be seen that the cells fit nicely into all the large and small indentations in the enamel surface. When the size of the epithelial cells is compared with that of the furrows in the enamel it is easy to understand that the small cells will have no difficulty in adapting themselves to the rather big furrows. There is an obvious difference between the tissue reaction to the rough tooth surface and that to the bacterial plaque, Figs. 2 and 4.[4] This shows that it is not the rough surface that irritates, but the bacteria or their toxins.

In this experimental series subgingival bacterial plaque was seldom found in the control pockets but often in the experimental pockets. The bacterial plaque was also found in isolated islands as in Fig. 4.[4] Isolated islands of subgingival calculus are hardly ever found in human autopsy material. The roughness of the tooth surface seems, consequently, to facilitate the retention of bacterial plaque. This is based on the observation[3] that it was impossible to retain bacteria in healthy pockets where no subgingival calculus was pres-

ent. A complete regeneration of the epithelial cuff and no subgingival calculus was the result in all controlled cases.

It is of interest to note that the healing was not impeded in the dogs with distemper although they were in an extremely poor physical condition. Toothbrushing did not seem to influence the healing of the tissue below the gingival margin in either direction in this experiment.

This experiment confirms earlier clinical experience that rough restorations cause irritation to the gingivae, but it shows that the irritation is not caused by the roughness itself but by the rough surface retaining bacterial plaque.

CONCLUSIONS

1. Rough surfaces do not in themselves irritate the epithelial cells with which they come in contact.

2. The irritating effect of subgingival calculus is caused by bacteria or their toxins.

3. A rough tooth surface facilitates the retention of bacterial plaque.

4. Restorations inserted below the gingival margin should be well polished.

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Erratum

The table printed below should be substituted for Table I now appearing in the article by Joseph C. Muhler and Donovan A. Weddle entitled "Fluorine Storage in Rats Receiving Different Fluorides in the Drinking Water at Varying pH" on page 897 of the December, 1955, issue of the JOURNAL.

TABLE I
COMPARISON BETWEEN DENTAL CARIES INCIDENCE AND FLUORINE STORAGE IN THE RAT RECEIVING THREE DIFFERENT FLUORIDES AT DIFFERENT pH'S IN THE DRINKING WATER
(Fluorine Concentration 20 μg per milliliter)

GROUP	NO. ANIMALS	pH OF DRINKING SOLUTION	MEAN WEIGHT (GM.)	WATER CONSUMED (ML./RAT/DAY)	NUMBER OF LESIONS	PER CENT REDUCTION	SKELETAL ANALYSIS (ASHED FEMUR)	
							F CONC. (μG/GM.)	TOTAL F (MG.)
Sodium fluoride	14	2	238	20.9	1.9	63	1,948	0.507
	17	4	211	22.0	6.1	10	2,041	0.473
	18	6	233	22.1	6.2	--	1,998	0.496
Stannous fluoride	15	2	212	21.3	2.5	51	1,923	0.495
	17	4	250	21.7	4.9	28	1,960	0.474
	16	6	227	22.0	5.6	6	2,373	0.573
Stannous chlorofluoride	20	2	248	20.7	2.4	53	948	0.263
	19	4	227	22.1	4.4	35	937	0.286
	17	6	200	22.2	4.7	22	1,232	0.286
Distilled water	16	2	254	22.1	5.1	--	42	0.110
	22	4	225	22.2	6.8	--	43	0.120
	20	6	233	22.2	6.0	--	109	0.144

A METHOD FOR THE COLLECTION OF LARGE QUANTITIES OF RAT SALIVA

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IN ORDER to obtain quantities of saliva adequate for a series of investigations on the caries-susceptible and caries-resistant strains of Hunt-Hoppert albino rats[1] it was necessary to devise an animal holder and arrive at a proper drug dosage to induce salivation. The necessity of preserving these rats and of retesting the same animals at a later date required a minimum of mortality.

A multi-unit animal holder was designed to allow quantity collection in the shortest period of time while maintaining the animals securely. Fig. 1 shows the holder empty and in use. It has the following measurements: over-all length, 33¼ inches; over-all width, 14¼ inches; height with cover, 8½ inches; number of sections, 8; width of each section, 4 inches; width of separations, 3/16 inch; head opening, 1½ inch diameter; width of bottle rack, 2 5/16 inches; individual bottle holder, 1¾ inch outside diameter; height of bottle, 3 inches; and angle of incline of unit, 8 degrees.

To obtain the required amounts of saliva during anesthesia and stimulation, it was found that forward inclination of the animals was necessary to prevent them from swallowing the saliva. Loss of saliva by this route was reduced to a minimum but not completely prevented. Eight degrees of inclination appeared to be adequate for good collection.

This unit can be used for rats of all sizes. The head opening can be adjusted by fastening circular cardboard cut-outs over the original aperture.

Wherever possible, Masonite was used to reduce weight and allow rapid cleaning. To reduce cleaning to a minimum the wood areas were hard finished by applying several coats of shellac with an electric spray; and in addition, a slip of brown wrapping paper was placed in each compartment. The size and weight of the unit enable one to carry it about and to set up operations in small quarters.

Pilocarpine nitrate (Merck), a sialogogue of long standing, was the drug used to induce salivation. However, the rat was very active after this drug was administered and yielded little, if any, saliva. The animal was anesthetized with Nembutal when attempts were made to induce salivation.

The initial results made it clear that the dosages for individual drugs, as stated by Griffith and Farris,[2] could not be employed with impunity when used in combination with another drug. Accordingly a study was made of the effect of various amounts of the 2 chemicals acting in combination.

Nembutal was administered first by intraperitoneal injection to bring about the anesthesia. This was followed by subcutaneous injection of the pilocarpine only after the anesthesia had taken effect. It was observed that

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after injection with the pilocarpine the animals became more relaxed than with Nembutal alone. Pilocarpine appears to enhance the effect of Nembutal.

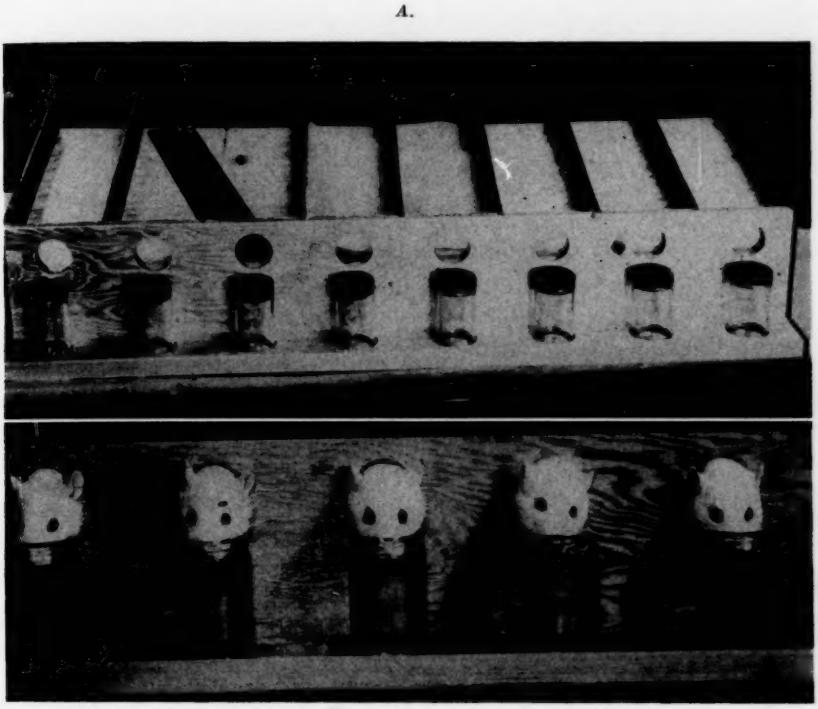

Fig. 1.—Photographs of multi-unit animal container. *A*, Multiple unit animal container (empty). *B*, Close-up view of animals salivating.

After many trials it was found that an initial injection of 20 mg./kg. was generally sufficient to produce the desired effect. If anesthesia did not occur, this was then followed by 5 mg./kg. increments up to a maximum of 50 mg./kg. This variation did not appear to be determined by sex, weight, or age. Pilocarpine was finally used, with satisfactory results, at a concentration of 5 mg./kg. This level produced salivation equal to that obtained with 100 mg./kg. and was equally effective for all ages and weights and for both sexes.

After both drugs were administered, the animals were placed in the holder and allowed to salivate until sufficient quantities were obtained. About 2 to 5 ml. of saliva could be collected from an adult rat within 20 minutes with approximately 10 per cent mortality.

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